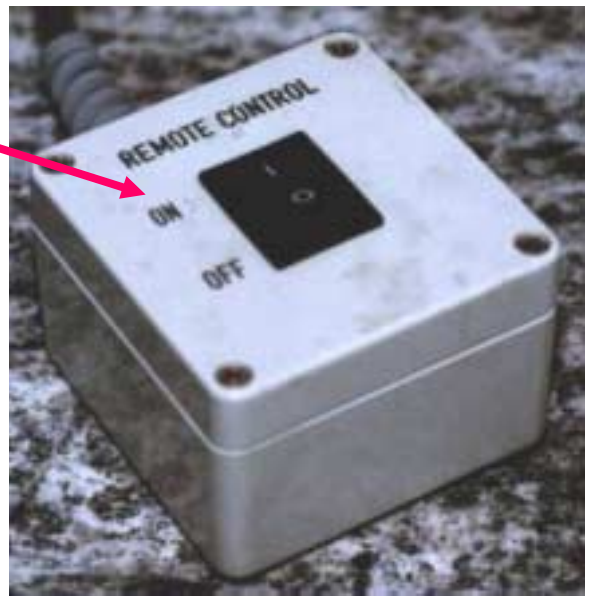
Overview

- Starting The Zeiss LSM 510 Microscope, Software And Laser
- Selecting An Objective And Focusing The Microscope
- Configuring The Laser Scanning And Detection For Confocal Image Acquisition
- Acquiring A Z And Time –Series
- Data Storage

Starting the Zeiss LSM 510 microscope, software and laser

- Turn on the remote control switch



- Switch on the mercury vapour lamp
  - Make a note of the number of lamp hours in the log book

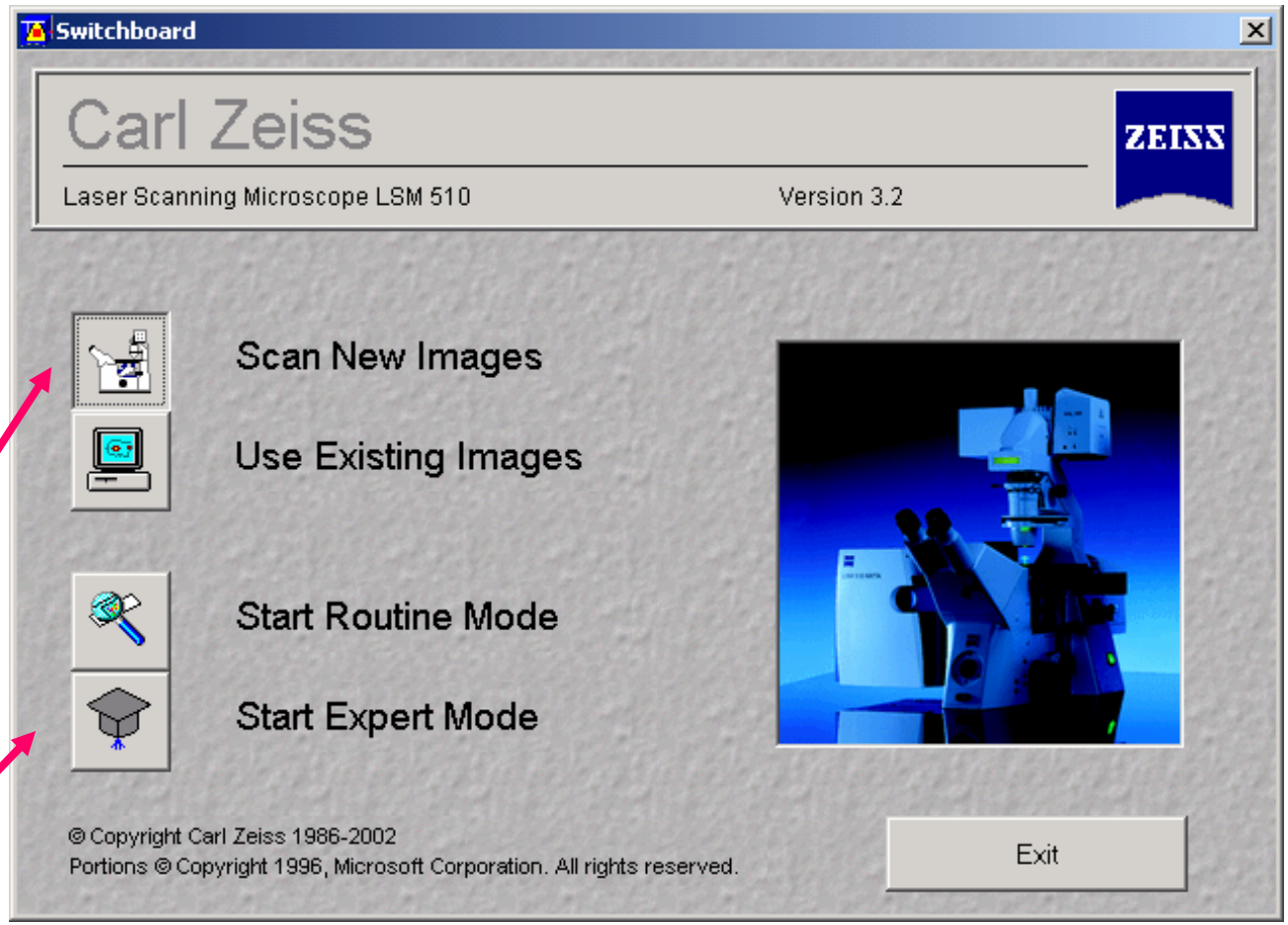
- Wait for the computer to boot up and Login with your own username and password

Starting The LSM 510 Software

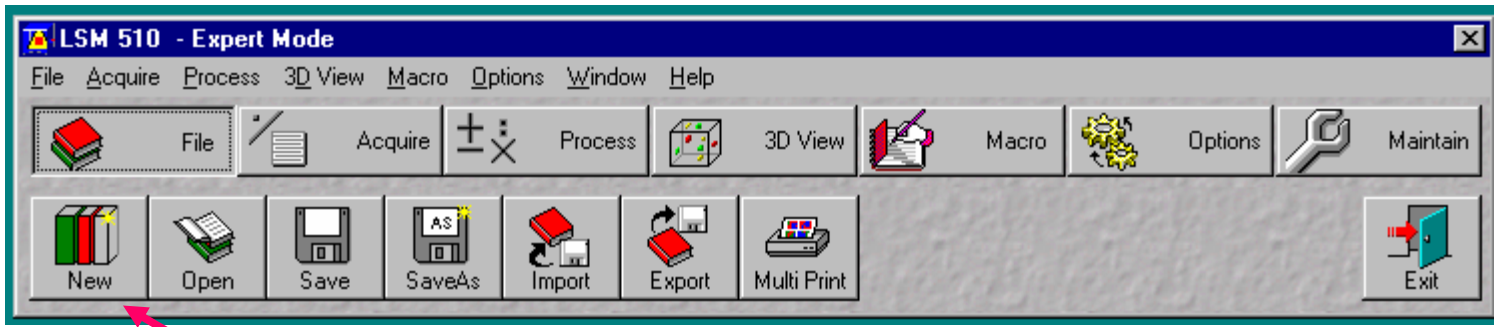
1. Double click the LSM 510 icon

2. Ensure “Scan New Images” is selected

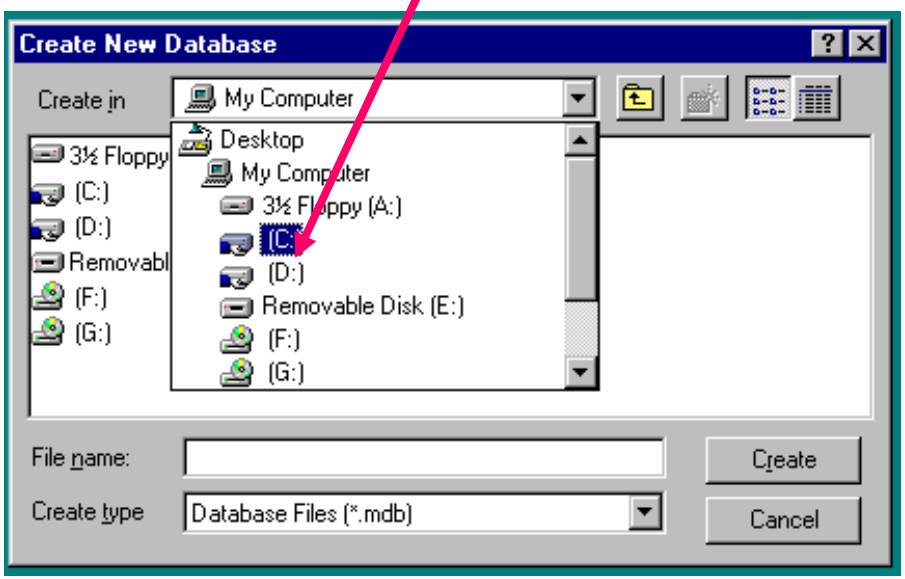
3. Select “Start Expert Mode”

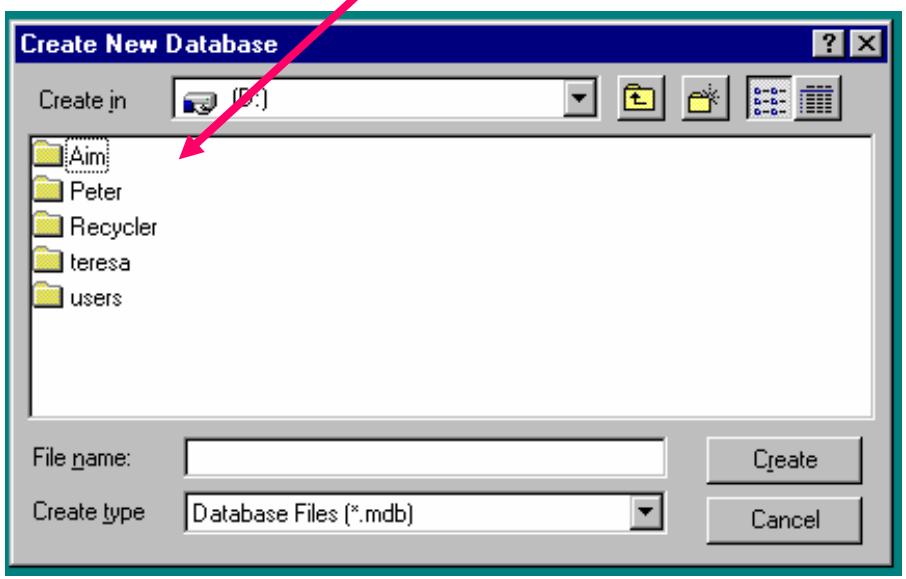


Creating A Database For Acquired Images

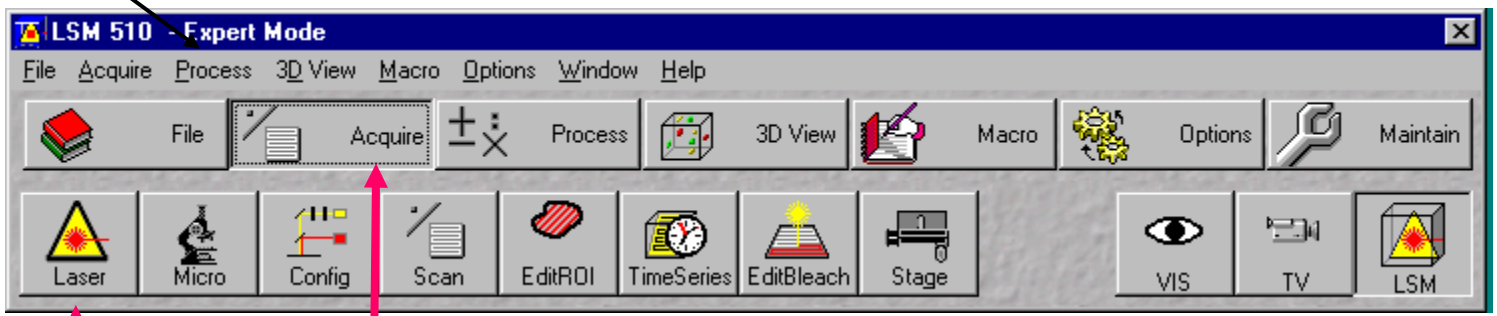


1. Select “New” database
2. Select drive D: from pull down menu
3. Create a new directory for each session





Turning On Lasers

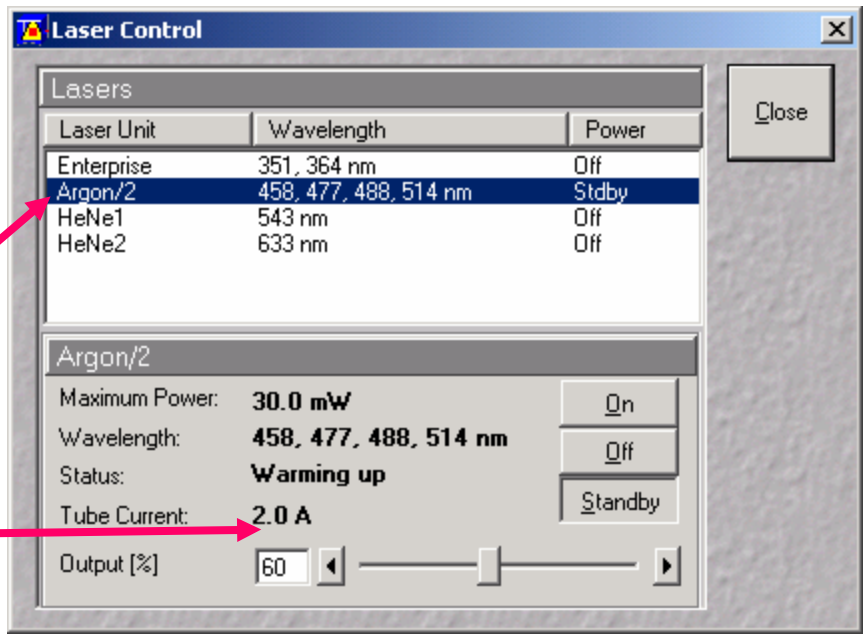


1. Select Acquire

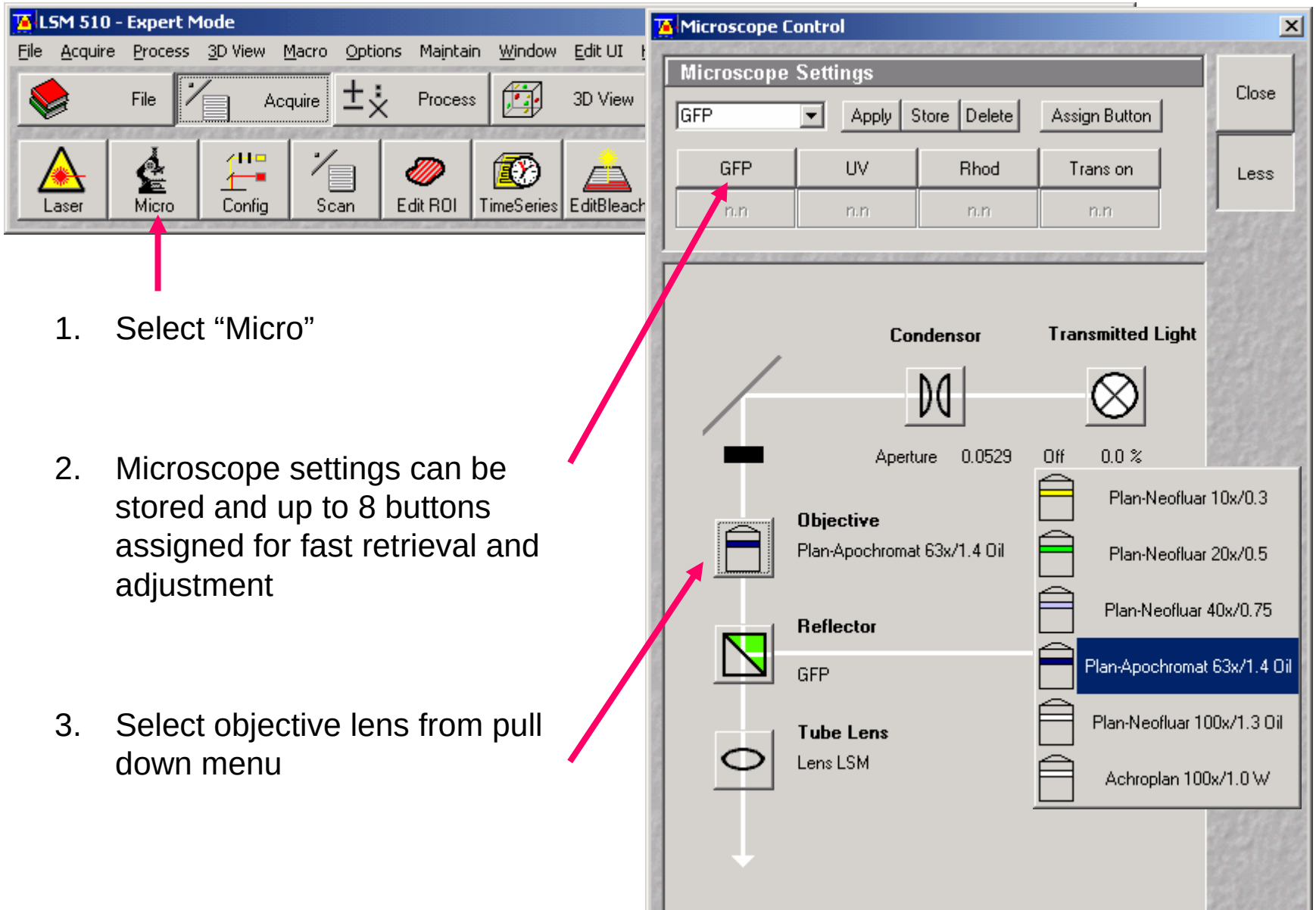
2. Select Laser

3. Switch required laser/s to Standby or On

4. The argon ion laser tube power must be set to 6.1 Amps



Microscope Eyepiece Viewing Or Laser Scanning

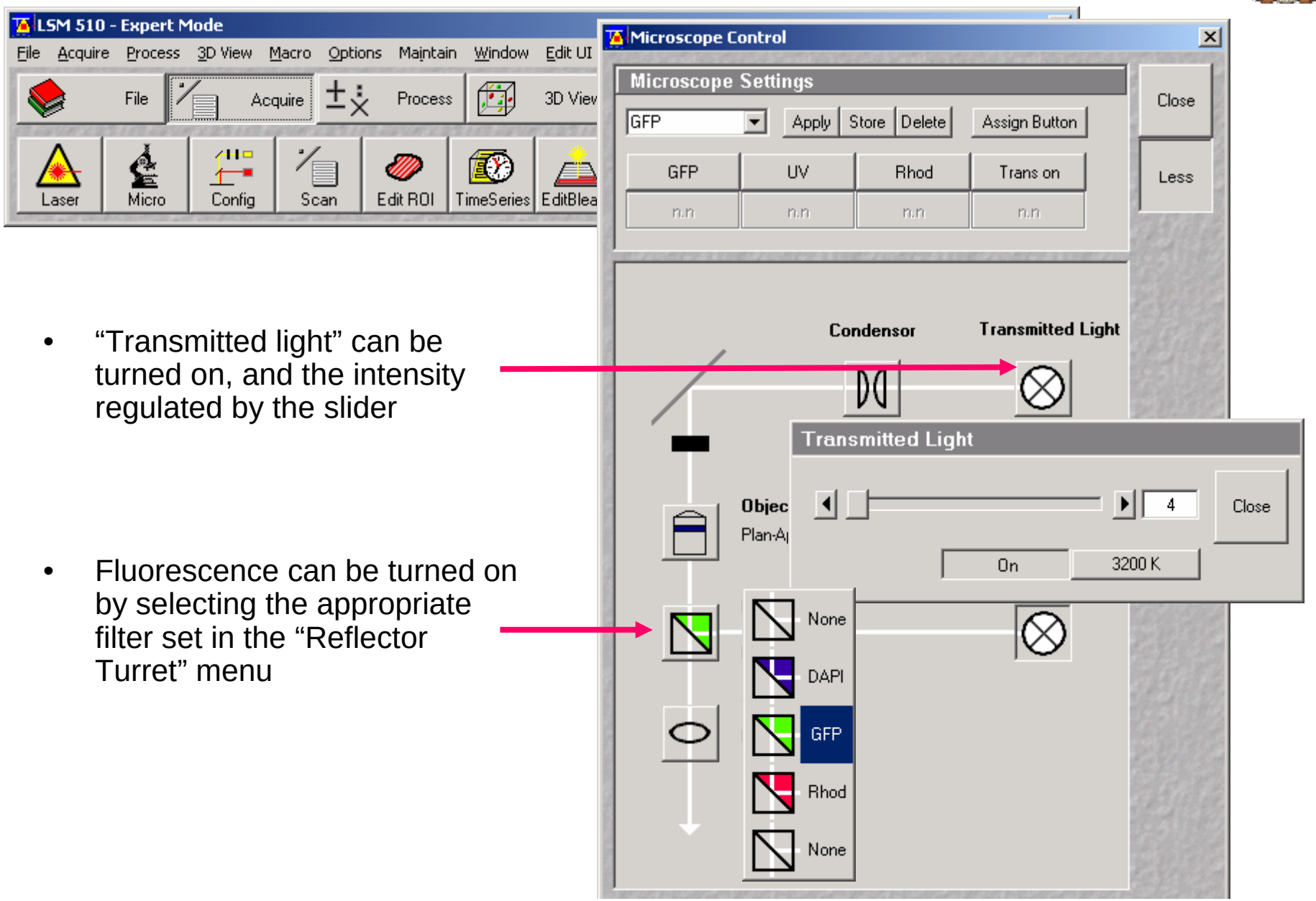


1. Select “Micro”

2. Microscope settings can be stored and up to 8 buttons assigned for fast retrieval and adjustment

3. Select objective lens from pull down menu

Focusing The Microscope In Fluorescence Mode



- "Transmitted light" can be turned on, and the intensity regulated by the slider

- Fluorescence can be turned on by selecting the appropriate filter set in the "Reflector Turret" menu

Configuring The Laser Scanning And Detection For Confocal Image Acquisition

Single Track

↓

Used for **single**, double and triple labelling

↓

Simultaneous scanning only

↓

Advantages

Faster image acquisition

↓

Disadvantages

Cross talk between channels

Multi Track

↓

Used for double or triple labelling

↓

Sequential scanning, line by line or frame by frame

↓

Advantages

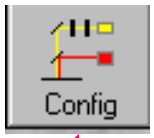
Dramatically reduces crosstalk

↓

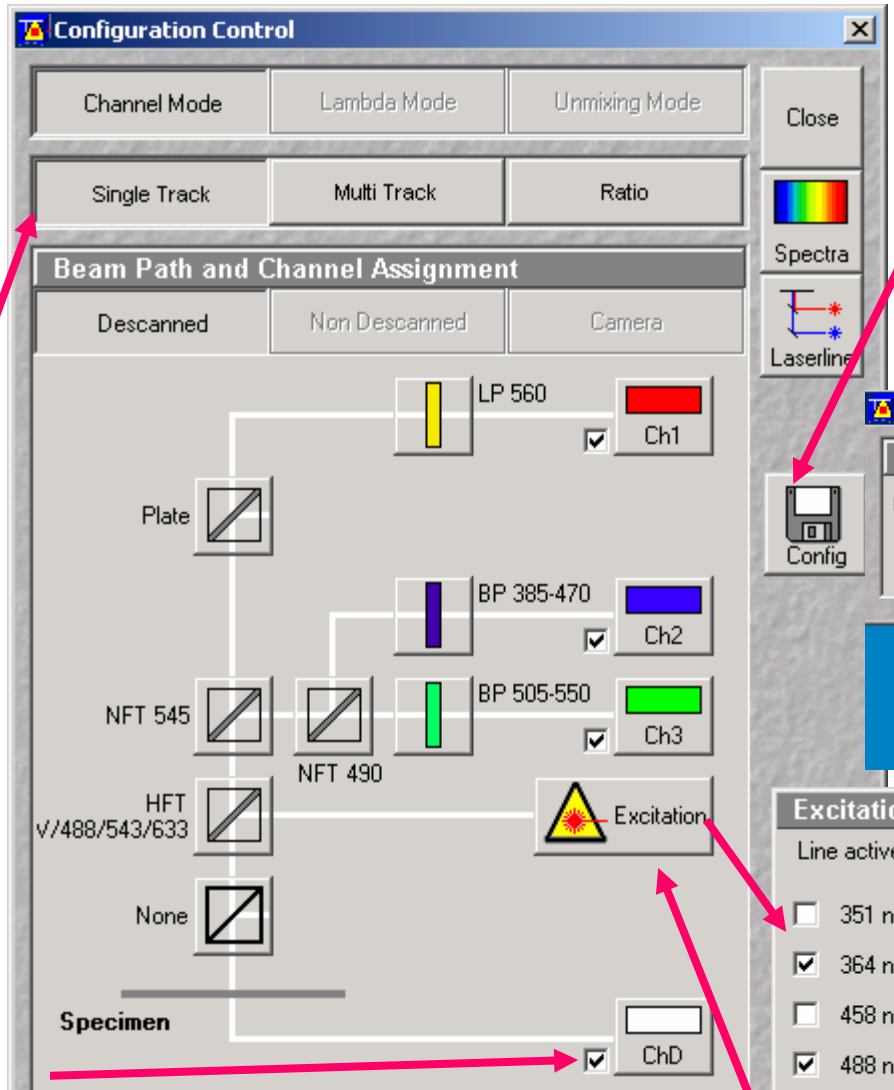
Disadvantages

Slower image acquisition

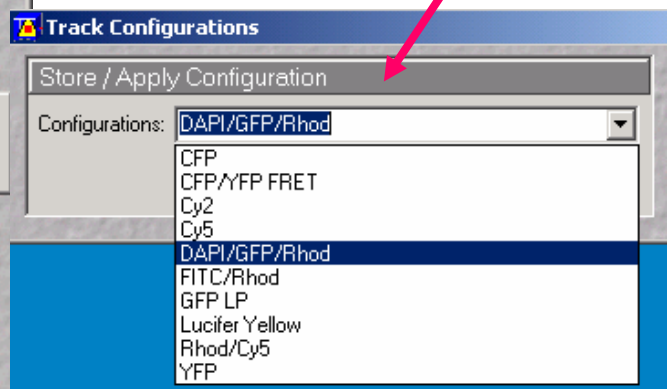
Configuration Of The Fluorescent Filters And Tracking

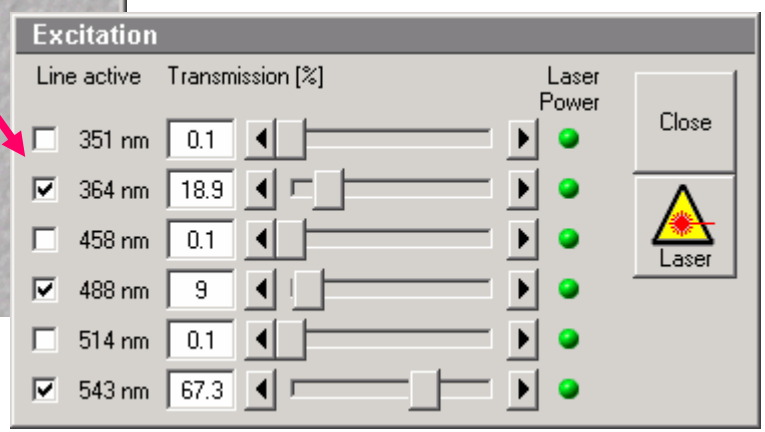


1. Select "*Config*"
2. Select Single Track



4. "the 'config' button opens the pull down menu to load/store track configurations

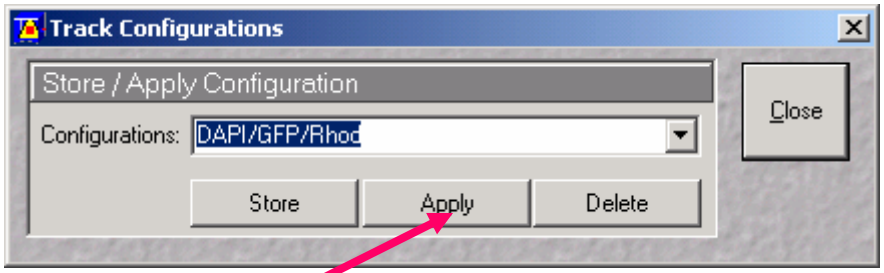




Transmitted light image can also be generated. Transmission channel is usually set to white colour.

3. click the excitation to select the laser and attenuation

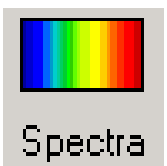
Applying The Configuration And Checking The Settings

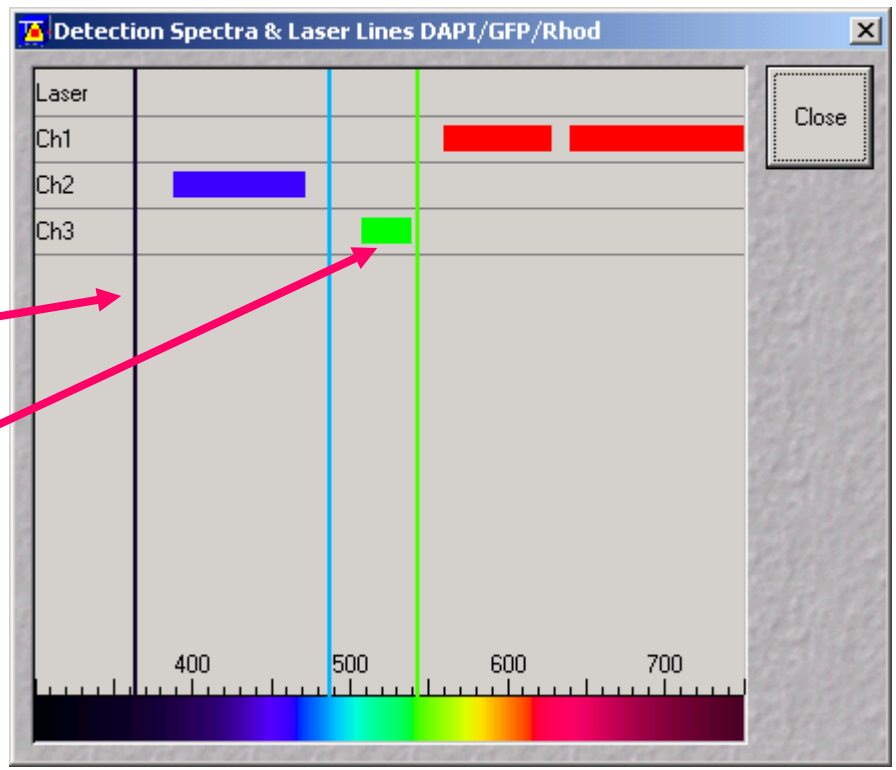


If you select store by mistake, it will ask you if you want to overwrite the configuration. Answer **NO**!

Each new login loads a predefined set of correct configurations.

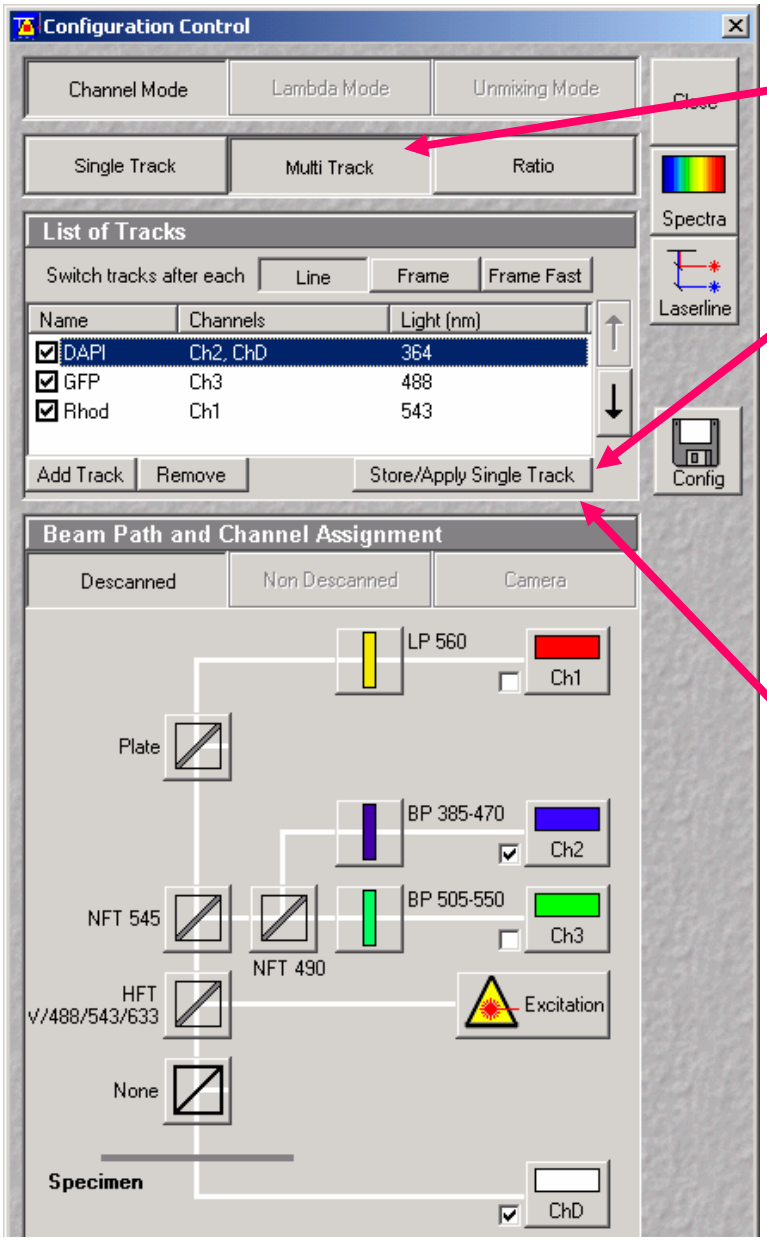
5. Select Apply
6. To check for correct settings, click the *Spectra* button



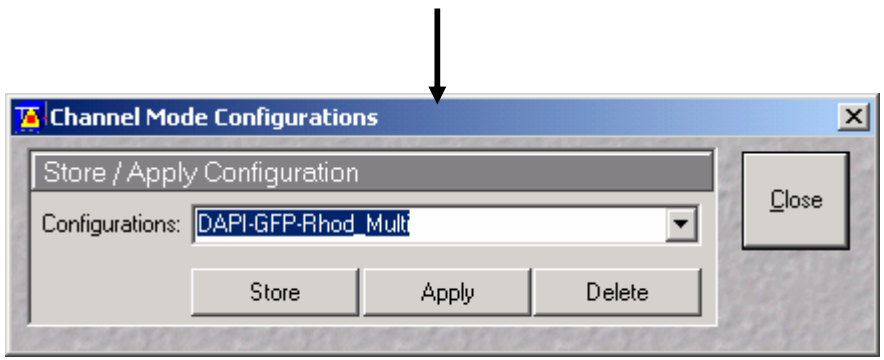


The spectra button opens a window to display the activated laser lines for excitation (colored vertical lines) and channels (colored horizontal bars)

Multi Track Configuration



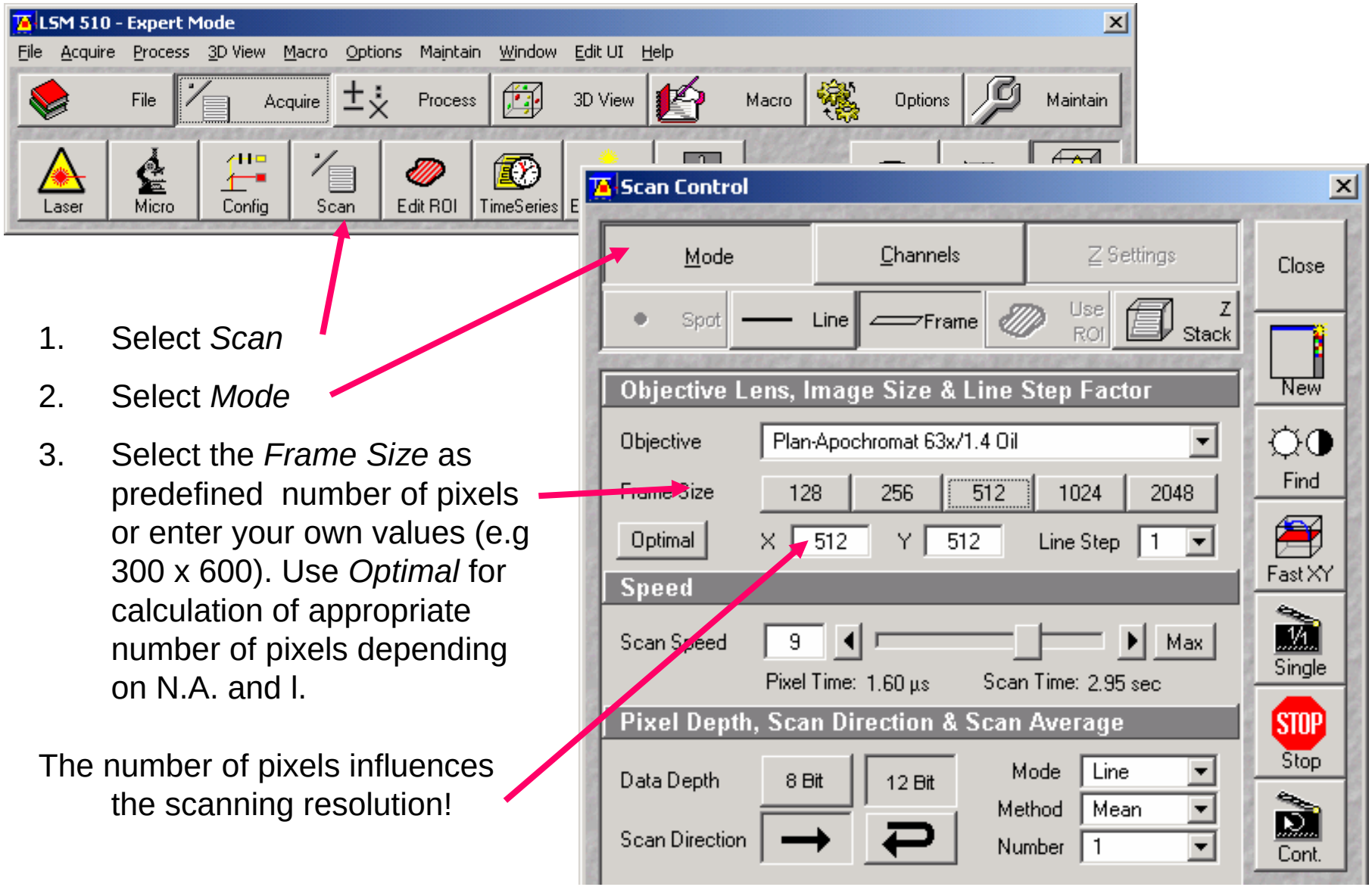
1. Select “Multi Track” for sequential scanning
2. Select Store/Apply
3. Select “DAPI-GFP-Rhod_Multi” from pull down menu



This is a button for single track configurations only.

Do Not Use Unless Sure!

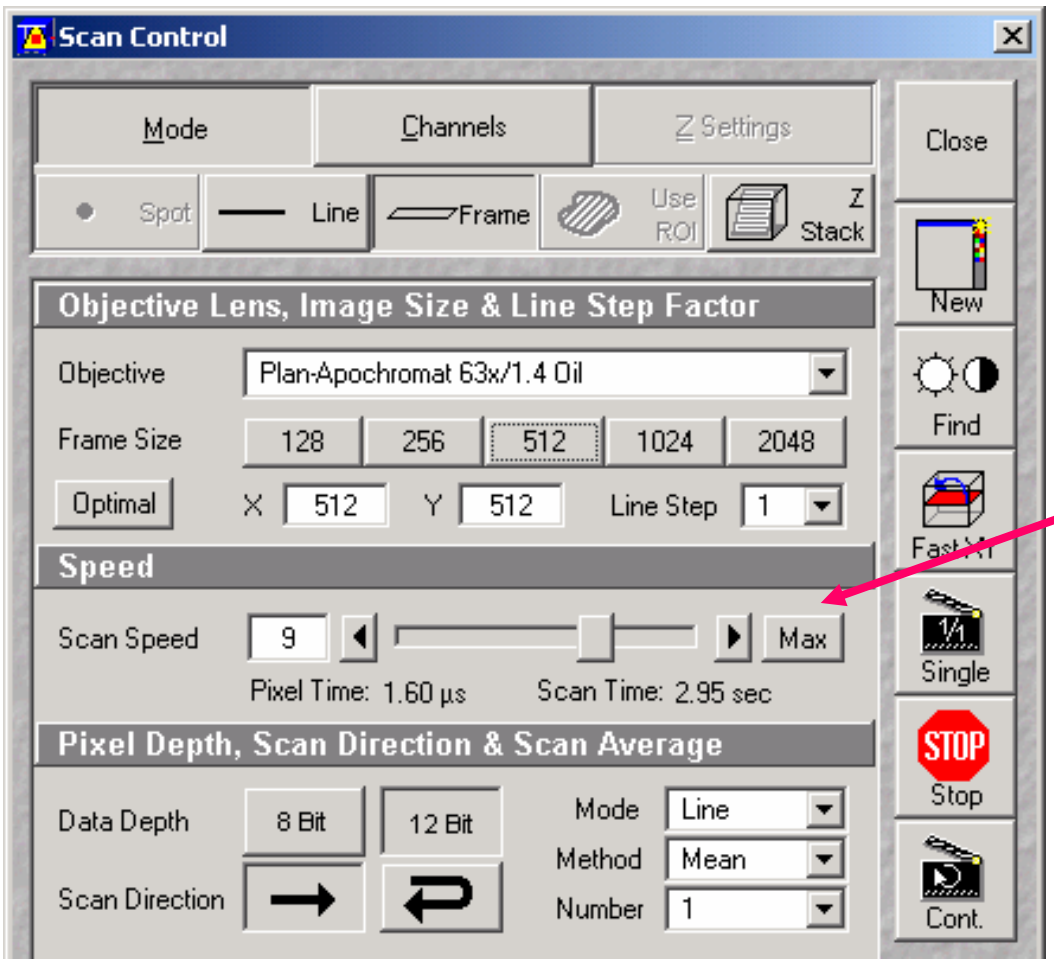
Setting Up The Scanning Parameters



1. Select *Scan*
2. Select *Mode*
3. Select the *Frame Size* as predefined number of pixels or enter your own values (e.g 300 x 600). Use *Optimal* for calculation of appropriate number of pixels depending on N.A. and l.

The number of pixels influences the scanning resolution!

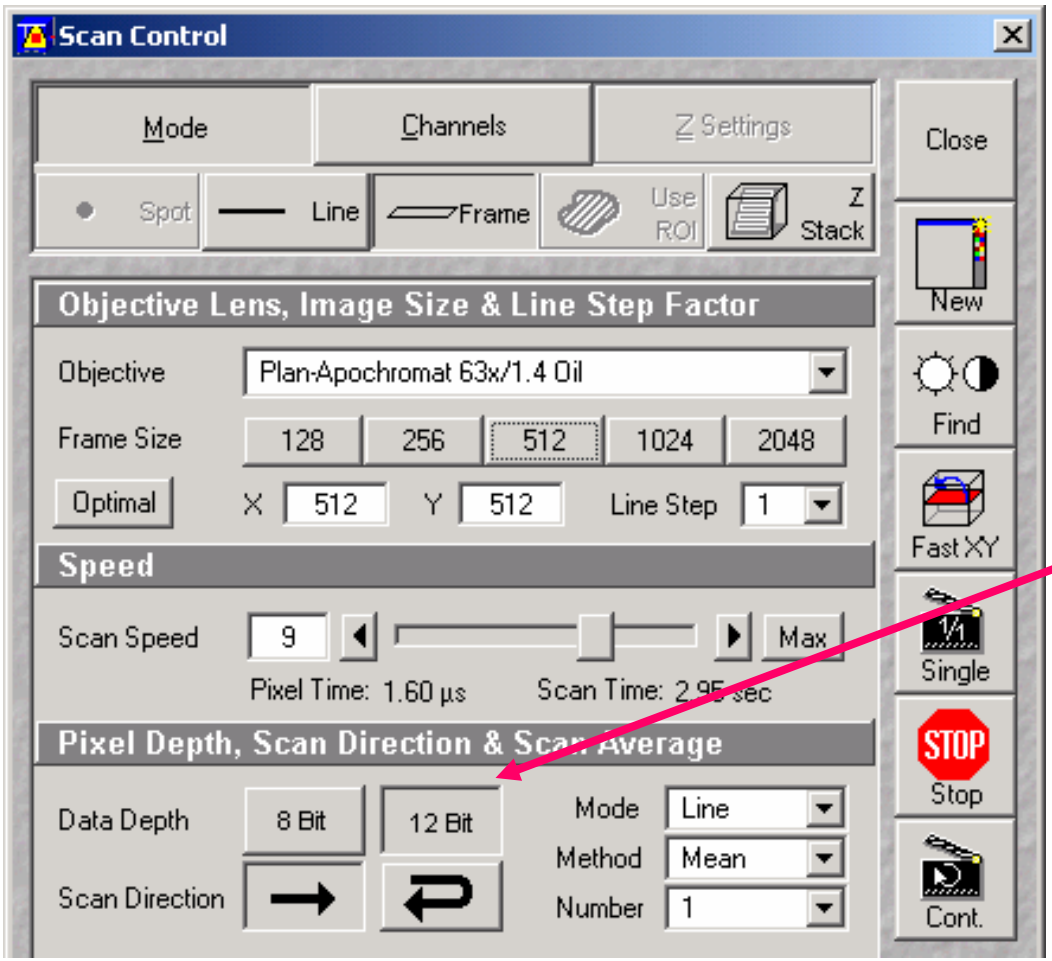
Entering Scan Speed



Enter the scan speed

- a higher speed with averaging gives the best signal to noise ratio.
- Scan speed 9 usually produces good results. Use 6 or 7 for superior images.

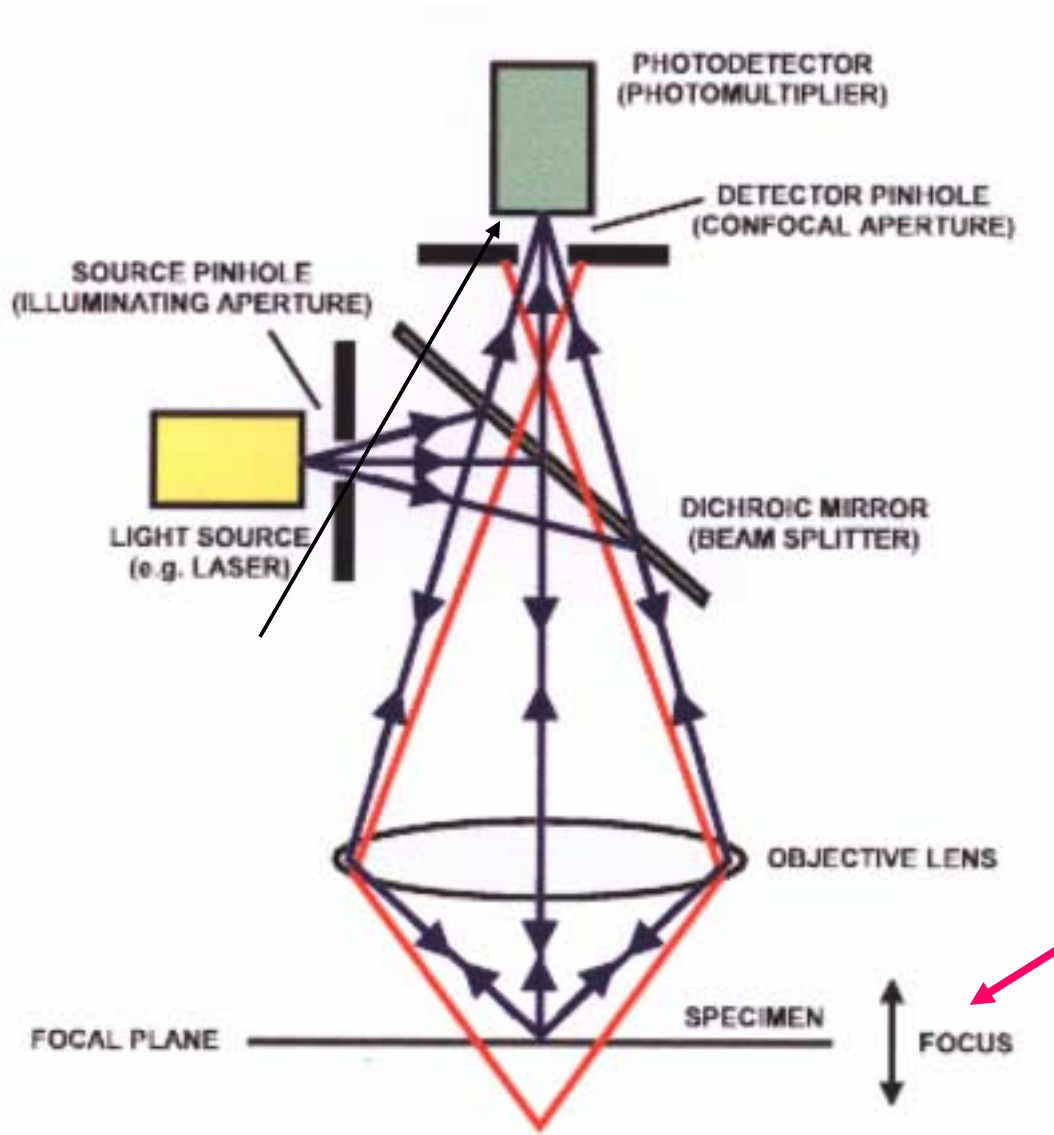
Setting Up The Dynamic Range (8/12 Bit Per Pixel)



Select the dynamic range

- 8 bit will give 256 grey levels, 12 bit will give 4096 levels.
- Photoshop 5 will import 12 and 16 bit images.
- Publication quality images should be acquired using 12 bit.

Confocal Microscope

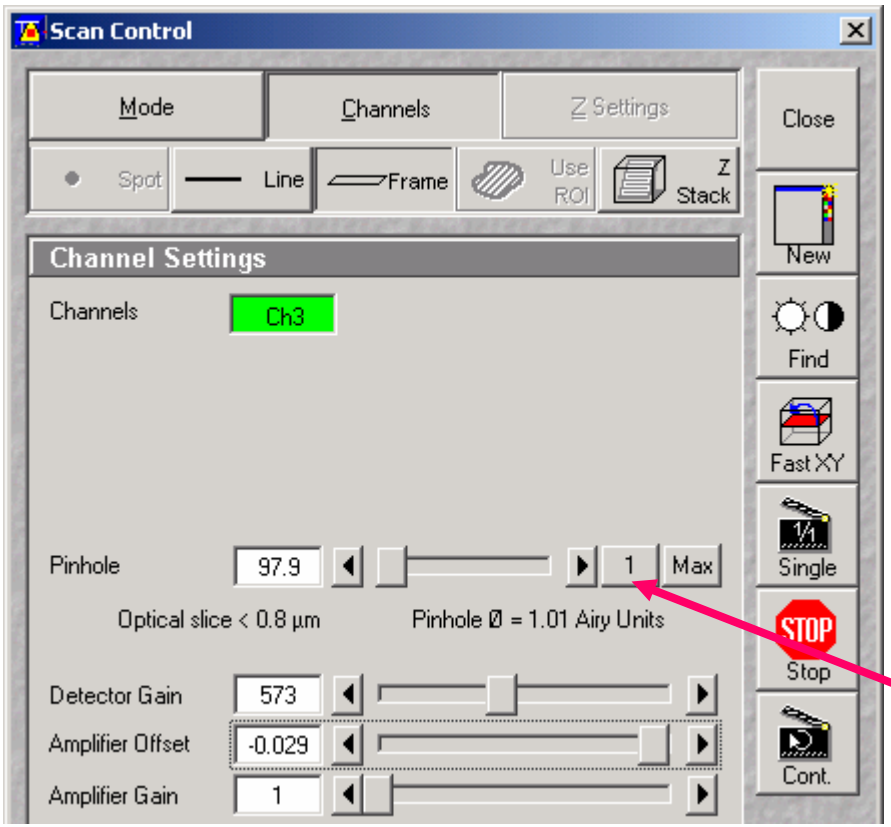


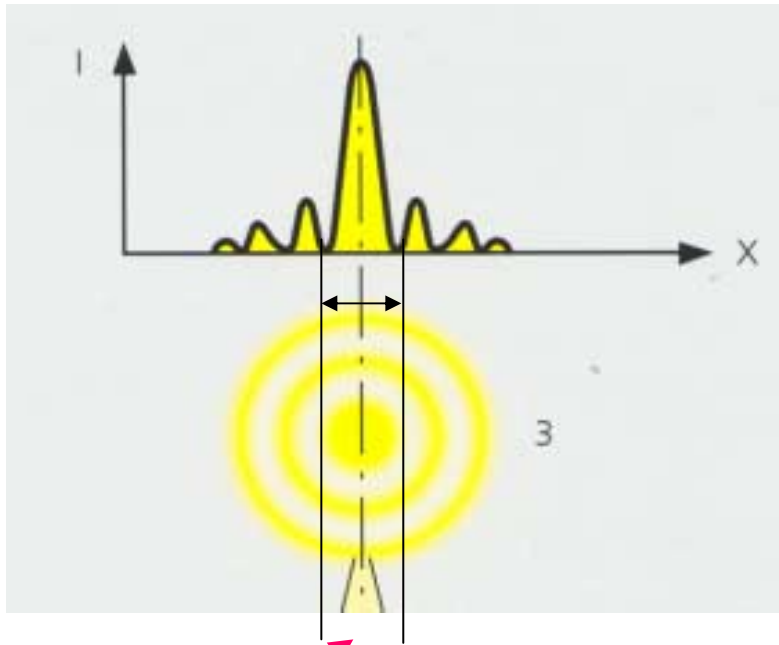
The depth of the optical section is dependant on:

1. Pinhole diameter (greater pinhole - thicker section)
2. Wavelength (longer wavelength - thicker section)
3. NA of objective (higher NA - thinner section)

Features above and below the plane of focus fall outside the pinhole and appear black - producing a true optical section.

Channel Settings - Adjusting Pinhole





Pinhole size =
1 Airy unit

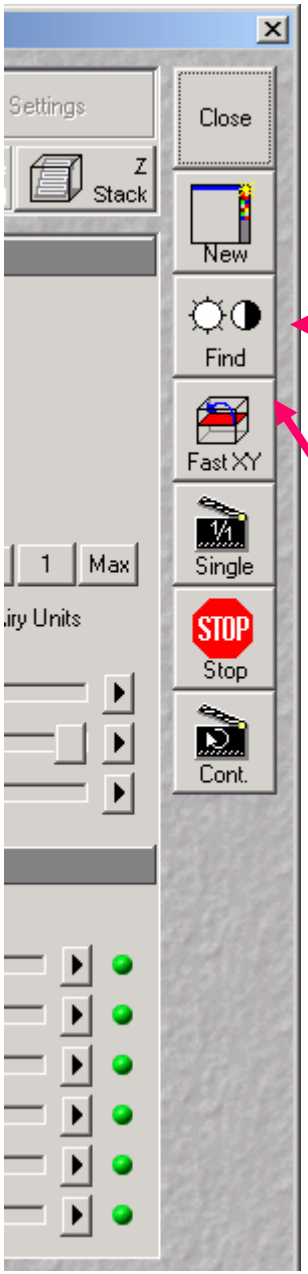
0.8 “Airy units” produces the best signal : noise ratio

Pinhole adjustment changes the “Optical slice”.

When collecting multi channel images, adjust the pinholes so that each channel has the same “Optical Slice”.

This is important for colocalisation studies.

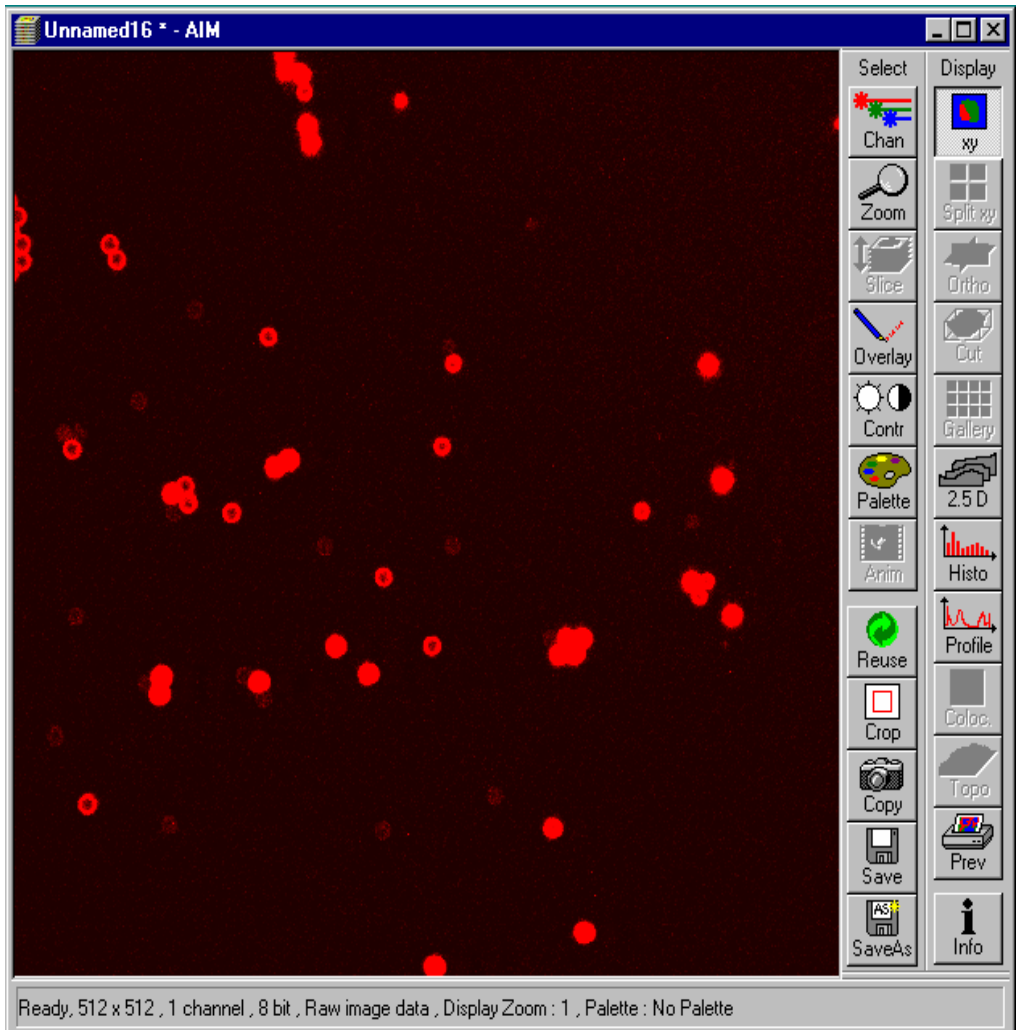
Starting The Acquisition of Images



1. Find automatically pre-adjusts detector sensitivity

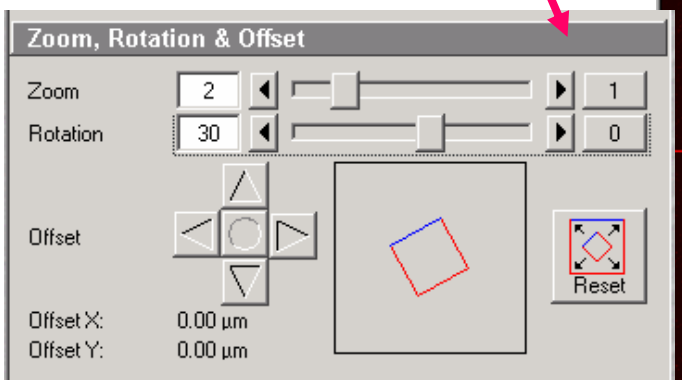
2. Select “Fast XY” for continuous fast scanning - useful for finding and changing the focus

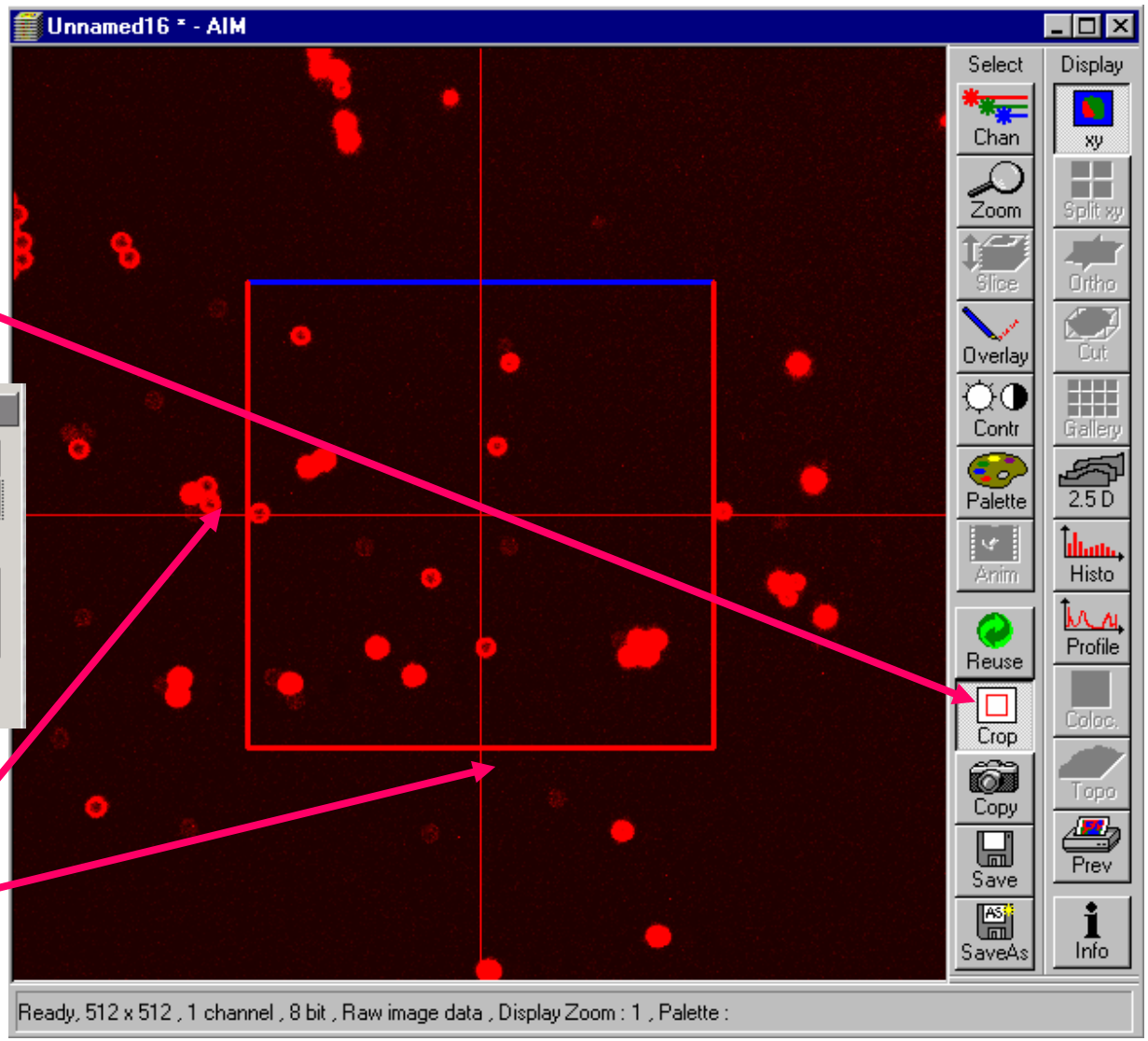
3. Stop



Optical Zooming

The level of zoom can be changed either by using the zoom control under “microscope”, or by selecting “Crop” on the image menu



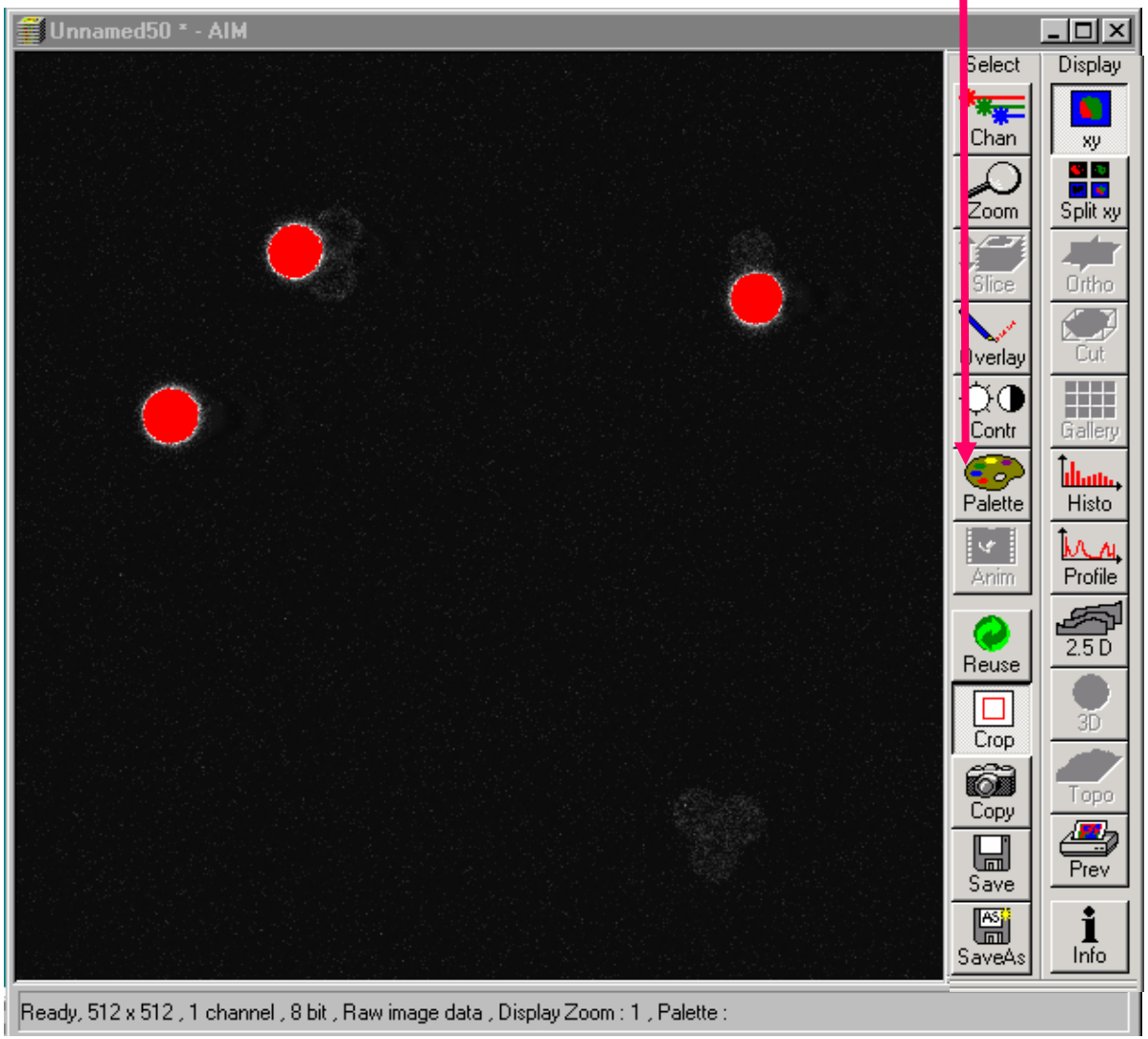


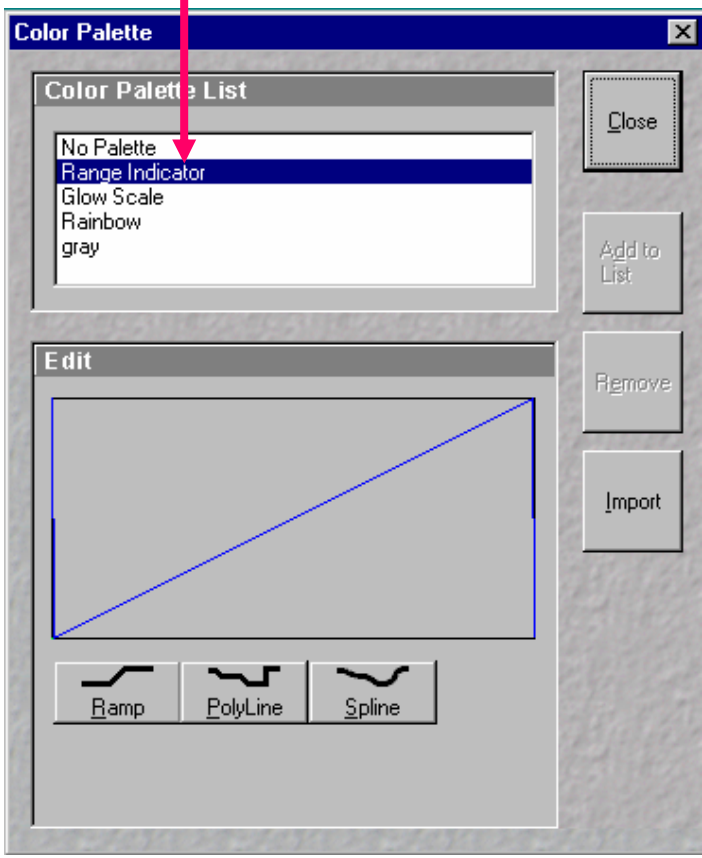
The image can also be rotated by selecting and dragging the bars

Selecting Gain And Offset - Choosing A Look Up Table

1. Select Palette

2. Select Range Indicator





Red = Saturation (maximum)

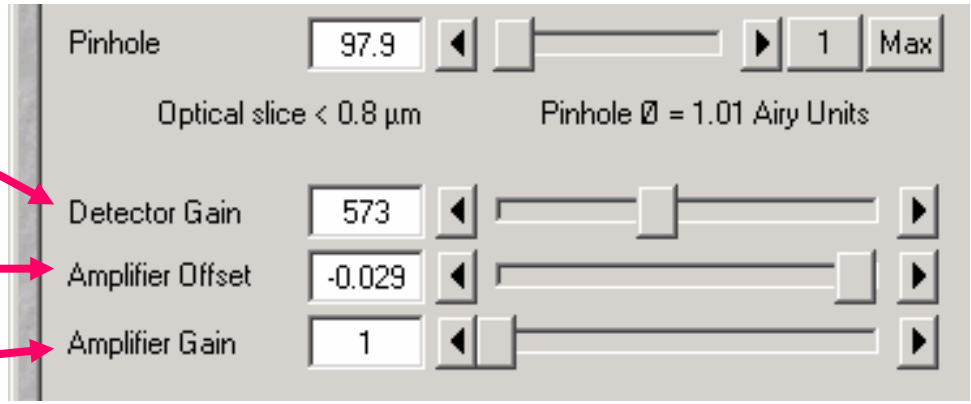
Blue = Zero (minimum)

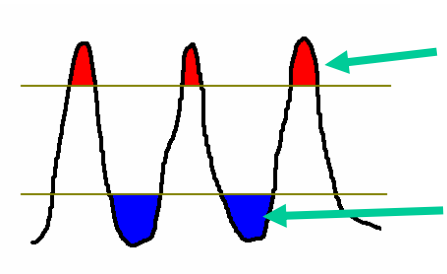
Scan Control - Setting Gain And Offset

“Detector gain” determines the sensitivity of the detector by setting the maximum limit

“Ampl. Offset” determines the minimum intensity limit

“Ampl. Gain” determines signal amplification



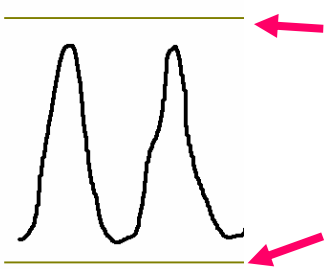


Saturation at the maximum

• reduce “Gain”

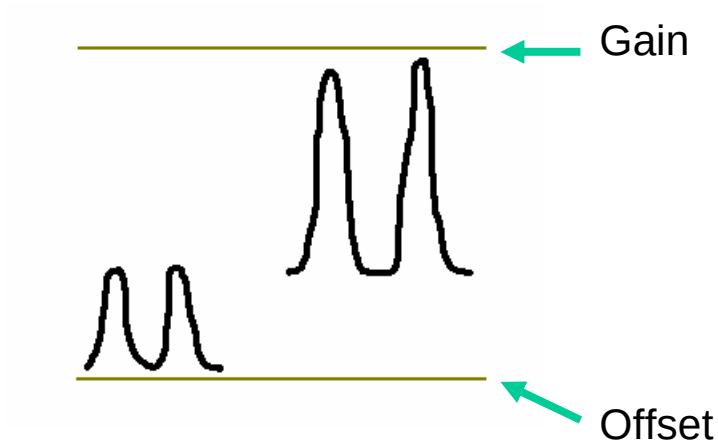
Saturation at the minimum

• increase “Offset”



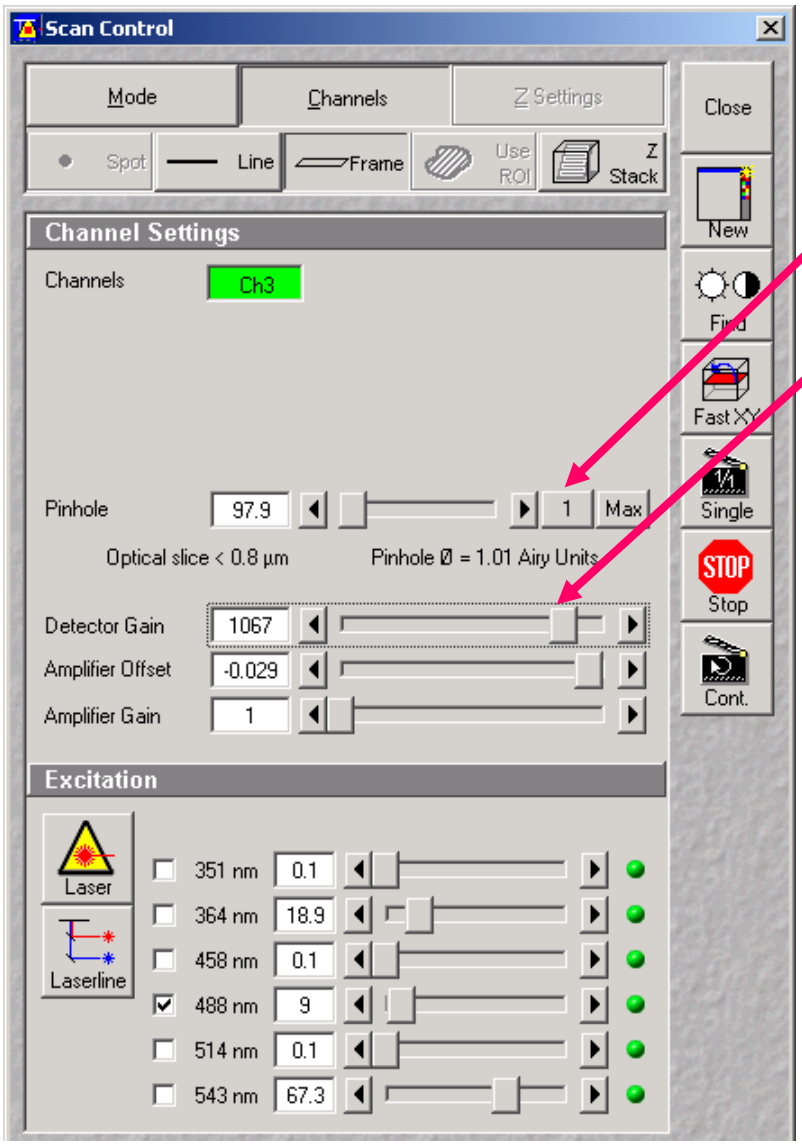
Gain set correctly

Offset set correctly

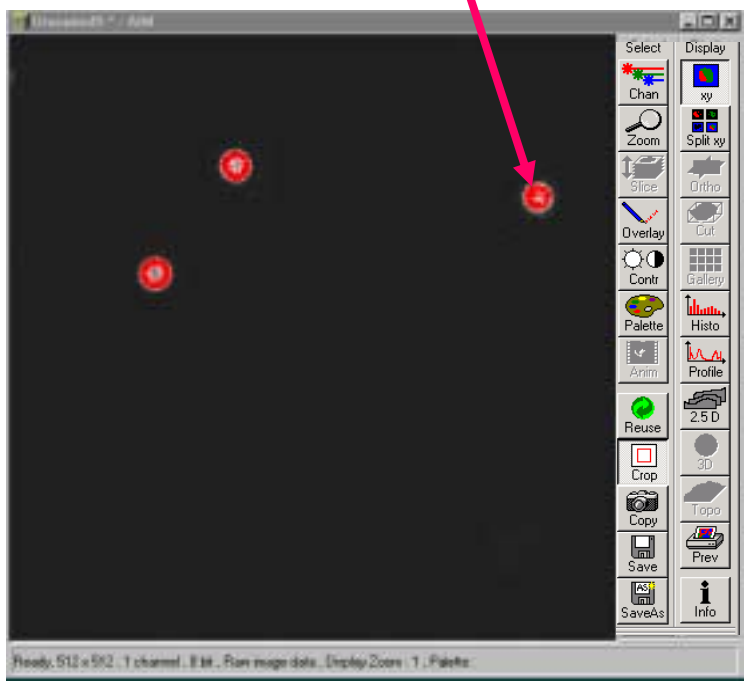


“Ampl. Gain”increases the whole signal, and the offset will need to be decreased.

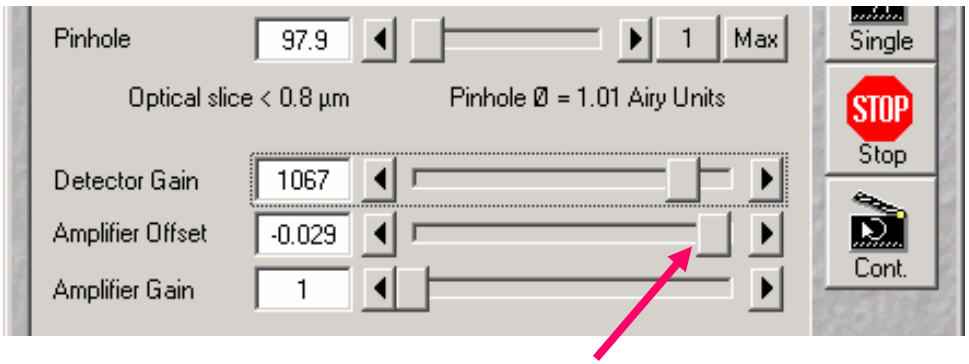
Adjusting The Laser Intensity



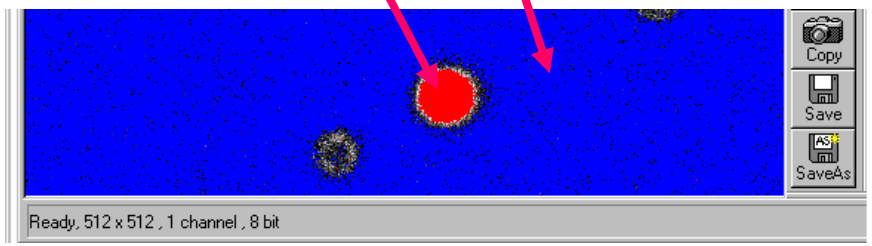
1. Set pinhole to 1 Airy unit
2. Set gain high
3. Reduce laser when image is saturated



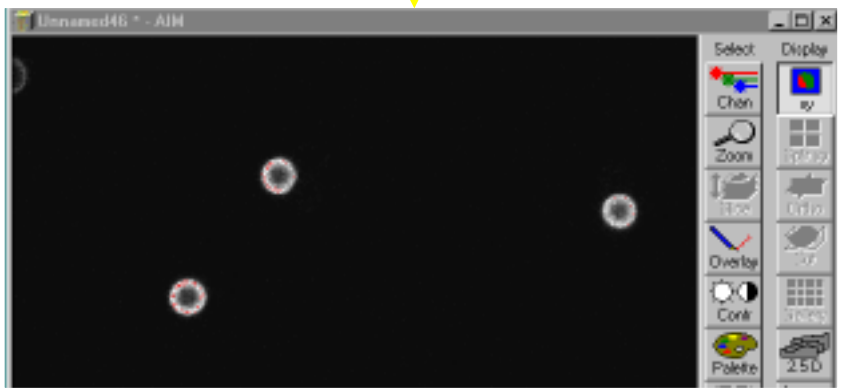
Adjusting Gain And Offset

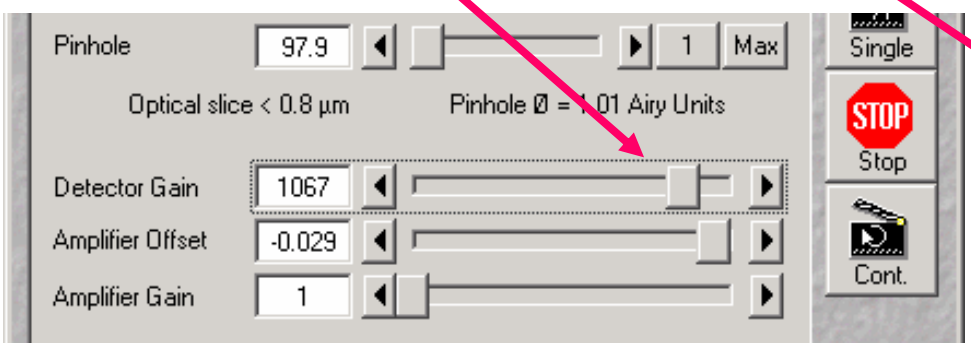


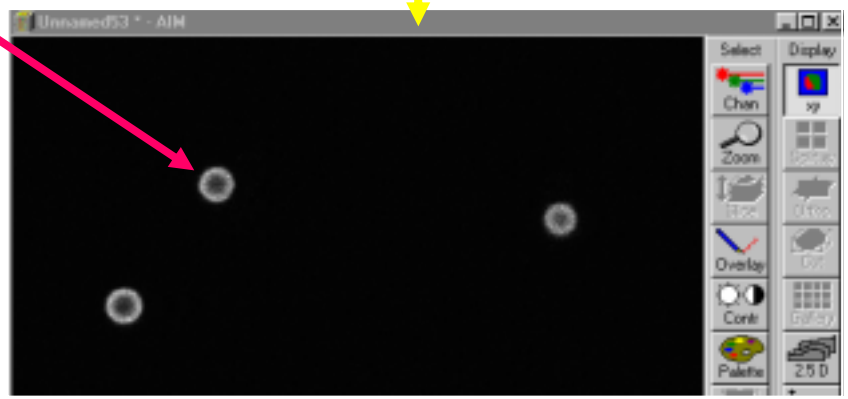
Both Gain and Offset saturated



1. Increase the Offset until all blue pixels disappear, and then make it slightly positive.
2. Reduce the Gain until the red pixels only just disappear.





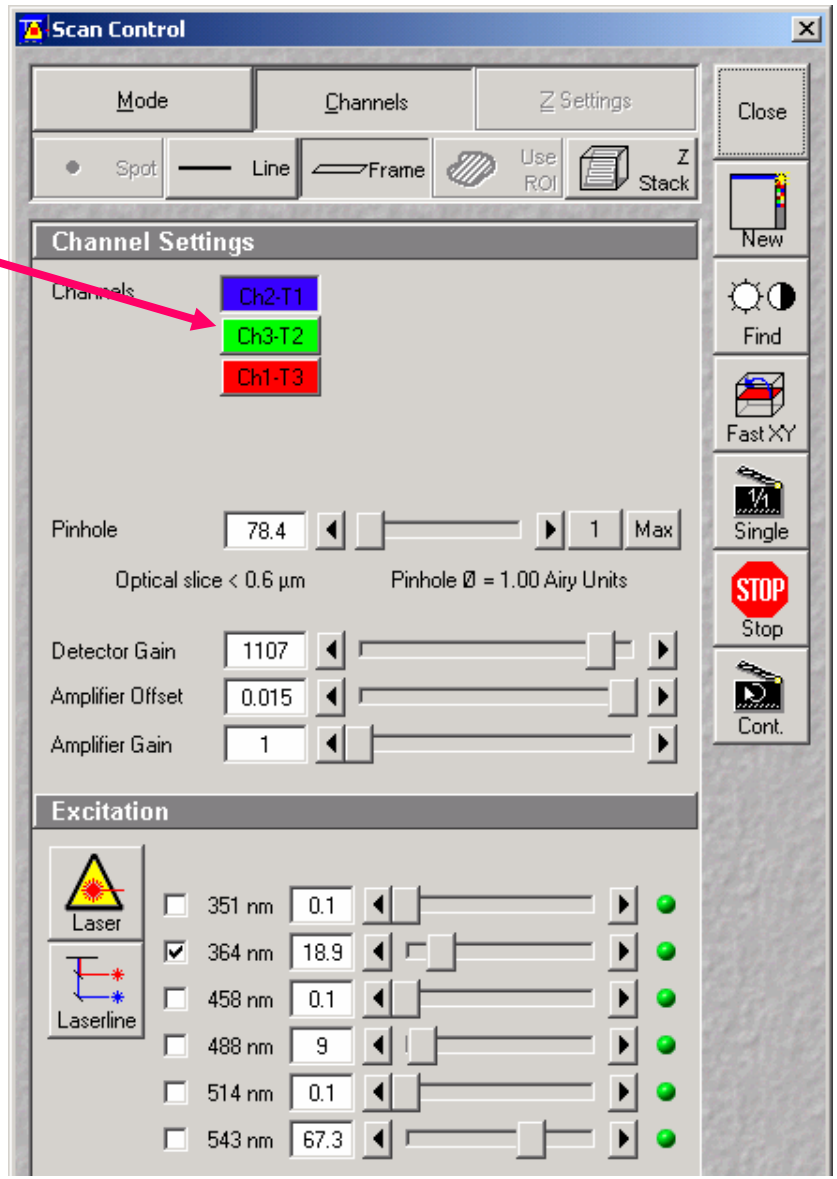


Adjusting The Laser, Gain And Offset Multitrack Configuration

Each channel is selected independently, and the laser power and other parameters are optimised as described in the previous slides.

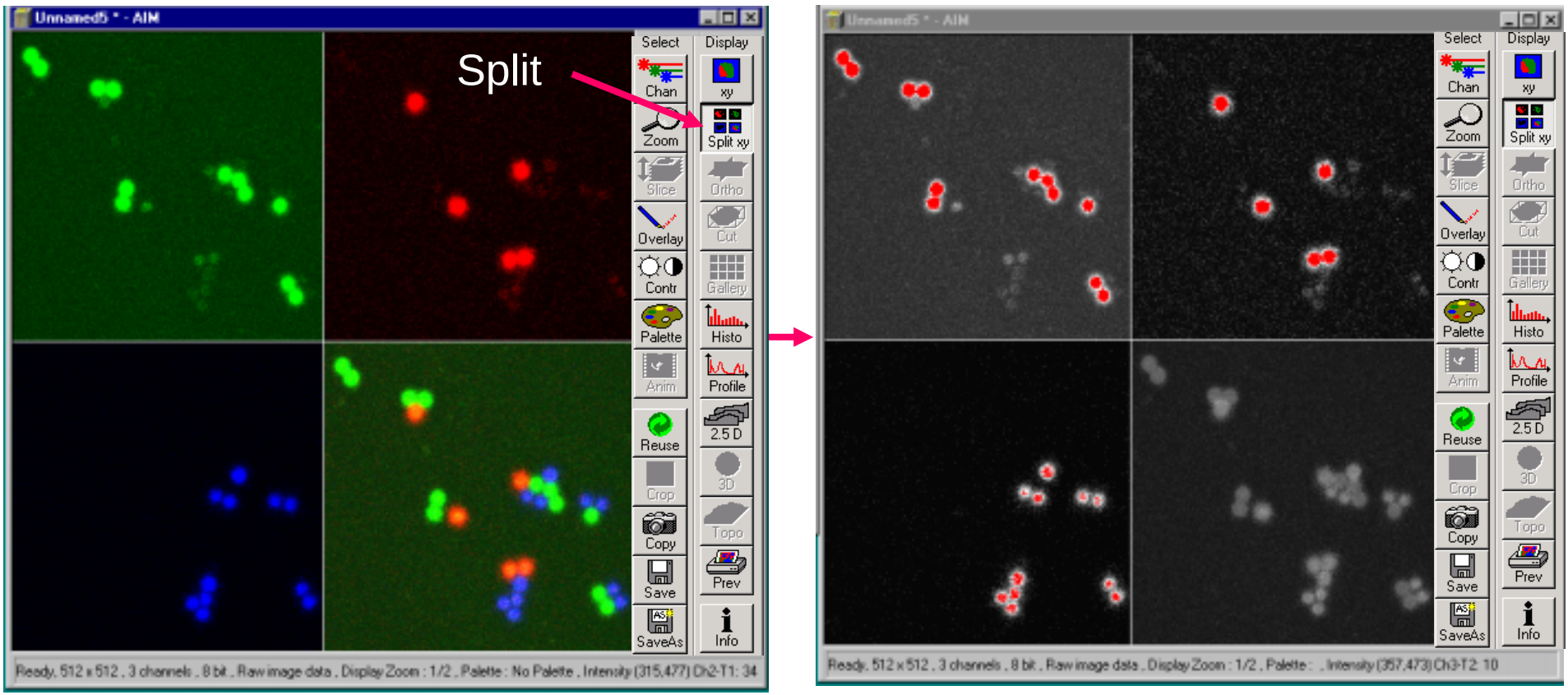
For accurate colocalisation, adjust the “Pinholes” so that each channel has the same “Optical Slice”

0.8 “Airy units” gives the best signal:noise ratio

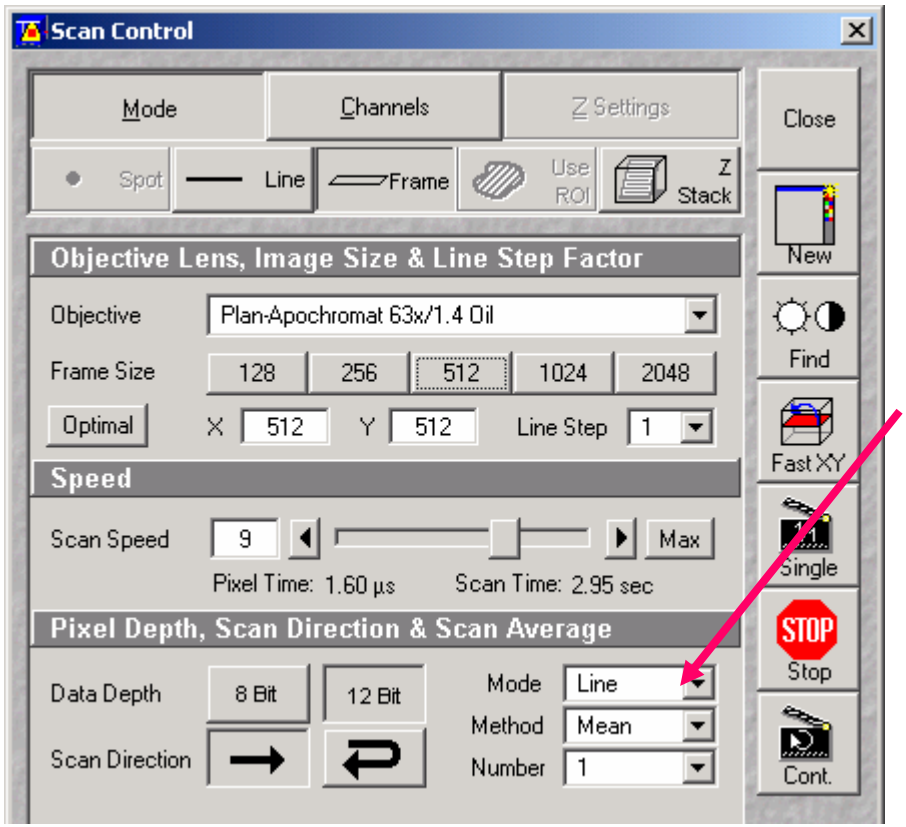


Setting Up Gain And Offset - Multi Track

1. Select *Split*
2. In *Palette*, select *Range indicator*
3. Select each channel separately under *Channels* and adjust the Laser, Gain, and Offset as described previously.



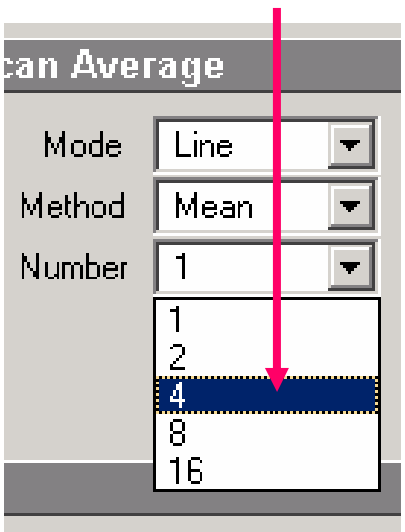
Line Averaging



Averaging improves the image by increasing the signal : noise ratio

Averaging can be achieved line by line, or frame by frame

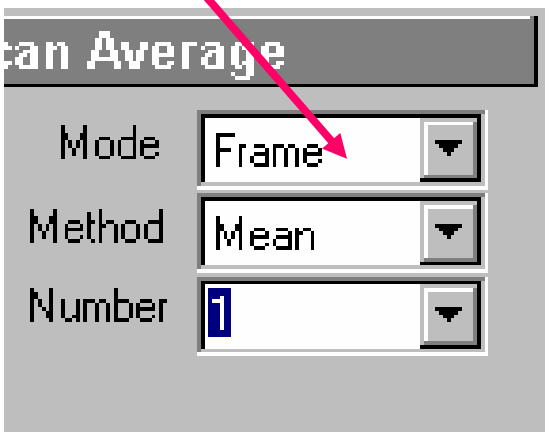
1. Select *Line* or *Frame*
2. Select number for averaging. the more the better (max 16) in this case, each line will be scanned 4 times

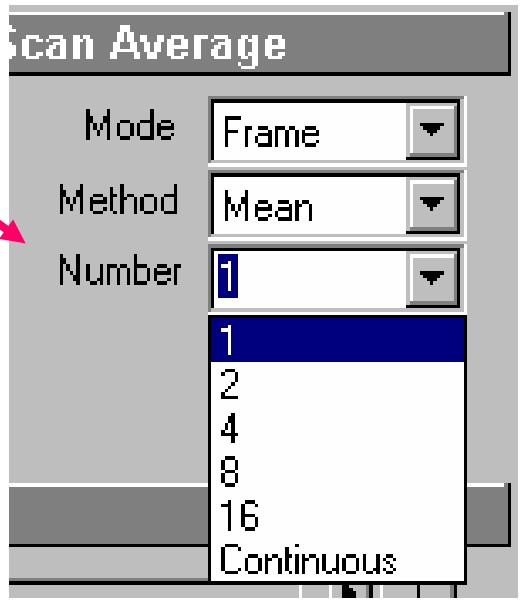


Frame Averaging

1. Select “Frame”

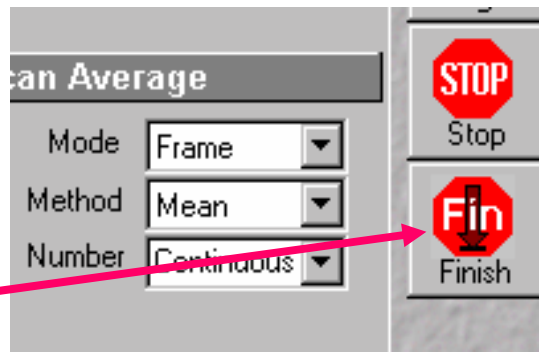
2. Select the number for averaging
   The more the better (max 16). Continuous averaging is possible in this mode





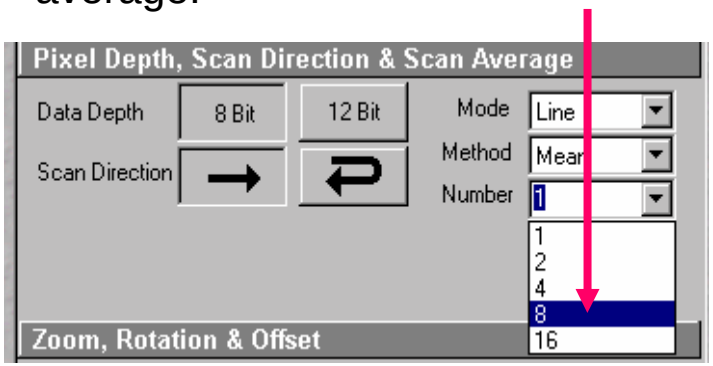
Frame averaging helps reduce photobleaching, but does not give quite such a smooth image. There is also a longer delay between each track when using “Multi Track”.

Continuous averaging has a “Finish” button which allows the scan currently in progress to be completed before stopping

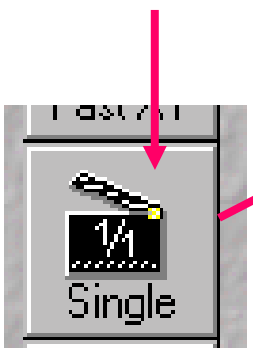


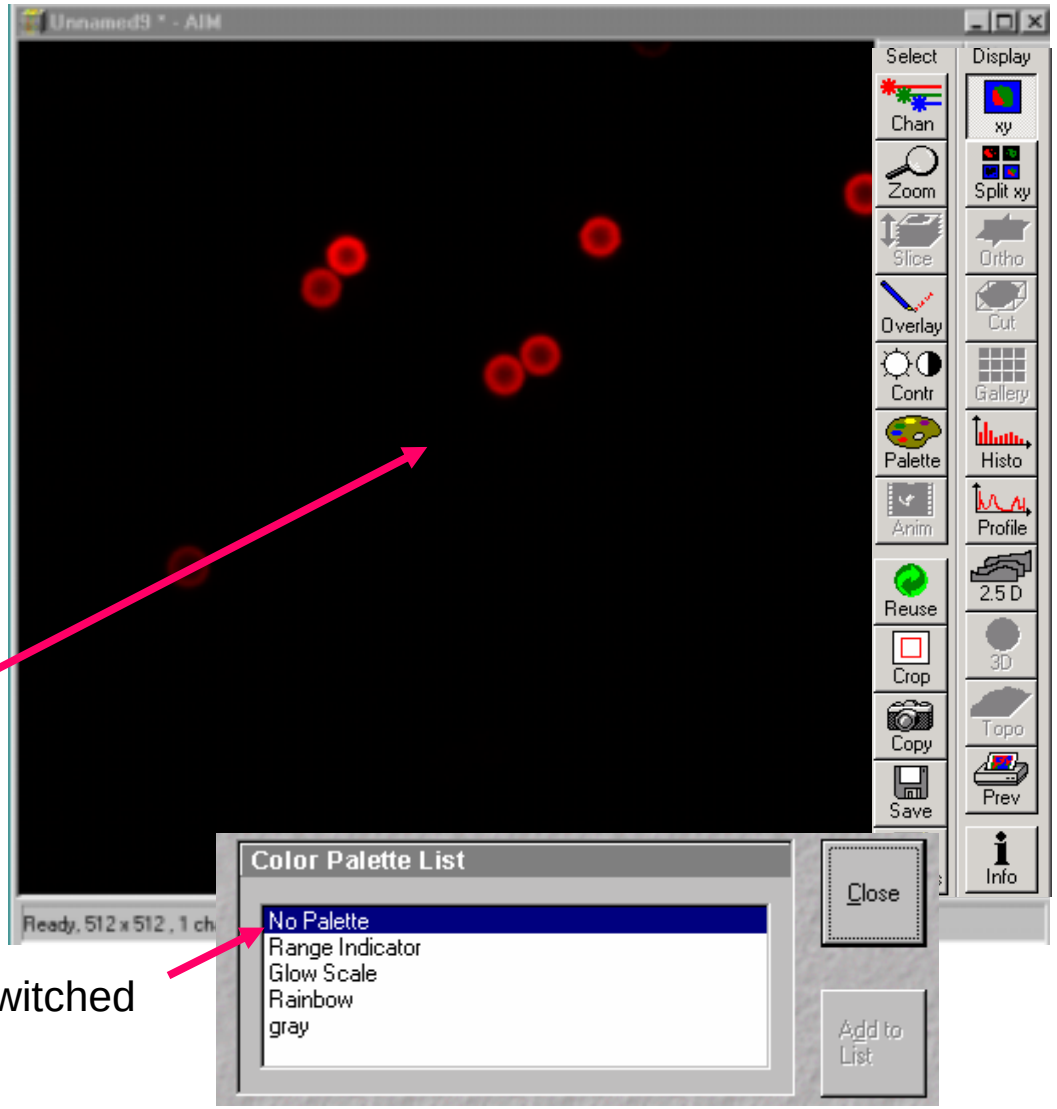
Collecting An Averaged Image

1. Return to *Mode*, and under *Scan Average* select the number for the average.



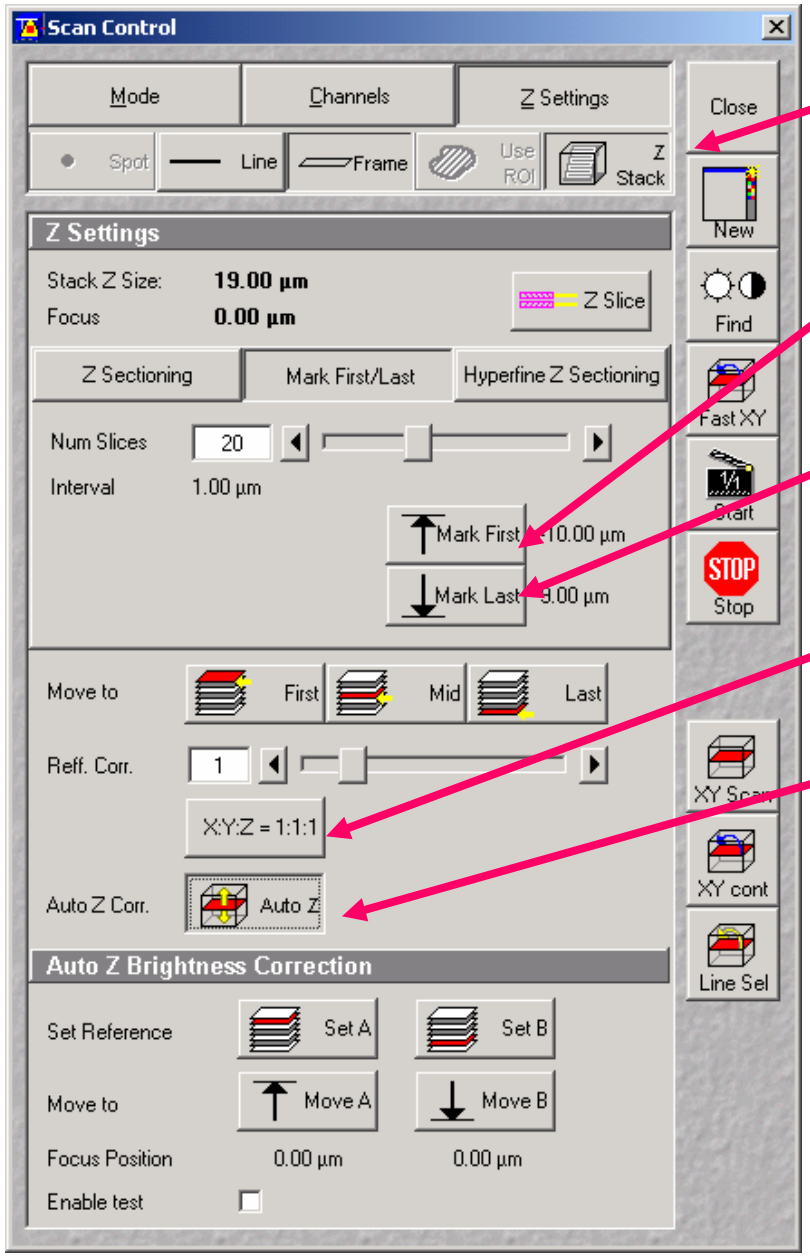
2. Under “Channels” select single”. An averaged image will be collected.





Range indicator switched off

Scanning A Z-Series Using “Mark First/Last”

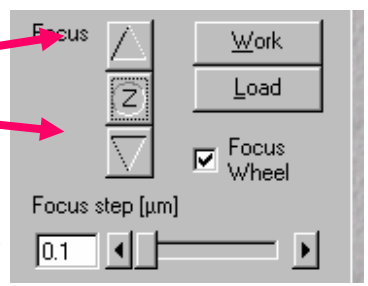


1. Select “Z Stack”
2. Start scanning using “Fast XY” or “XY Cont”
3. Keep your eye on the image and move the focus to the beginning of the Z series - select “Mark First”
4. Move the focus back in the opposite direction to the end of the Z series, and select “Mark Last”
5. X:Y:Z sets the Z-interval so that the voxel has identical dimensions in X, Y, Z.
6. with Auto Z Corr., *Detector Gain*, *AOTF*, *Ampl. Offset and Ampl. Gain* can be varied between two (A, B) freely selectable slices of a stack

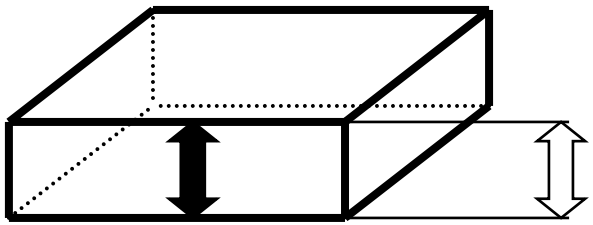
Focusing can be achieved manually (preferred), or using “Stage” on the LSM menu

Focus

Increment



Confocal Z-sectioning
Number Of Sections For Correct Sampling

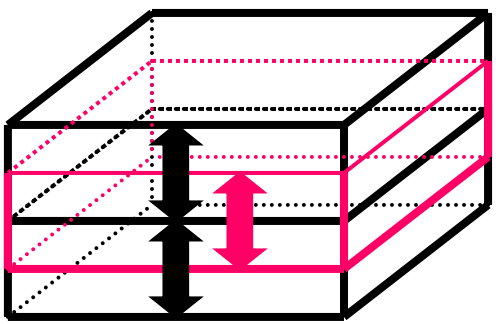


Optical thickness d depends on:

- wavelenght λ
- objective lens, N.A.
- refractive index n
- pinhole diameter P

$$d \sim P\, n\, \lambda \,/\, (N.A)^2$$

~ 0.5 μm @ 63x1.4

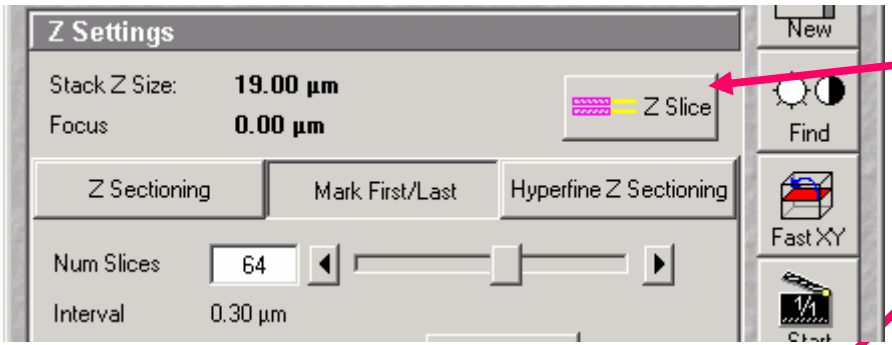


Optimal: (no missing information @ minimal number of sections)

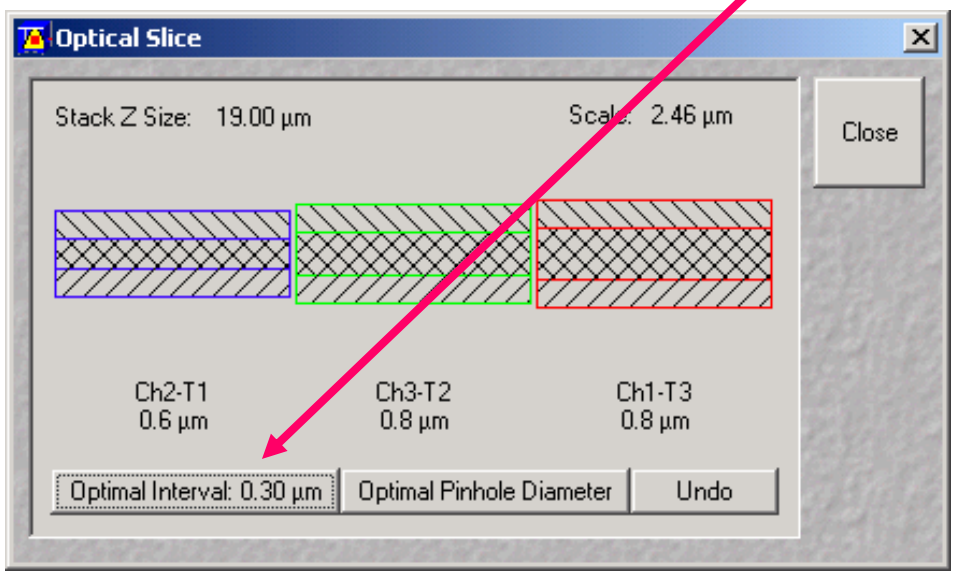
slices overlap by the half of their thickness

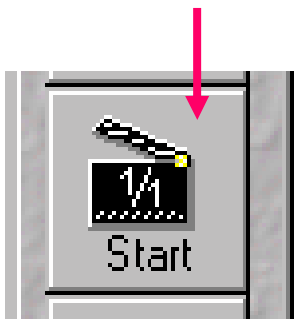
„Nyquist-“ or Sampling- Theorem

Z Stack - Number Of Slices And Increment



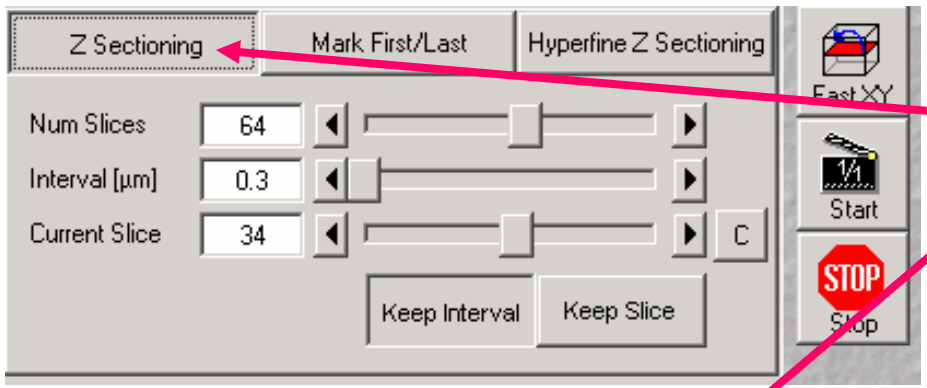
1. Select Z slice - the window *Optical Slice* will appear
2. Select *Optimal interval* the computer will calculate the optimum number of sections
3. Select “Start”



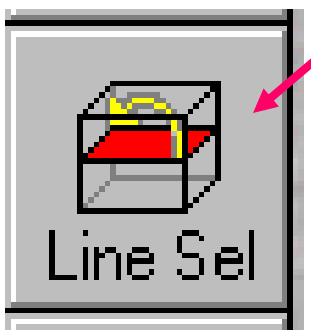


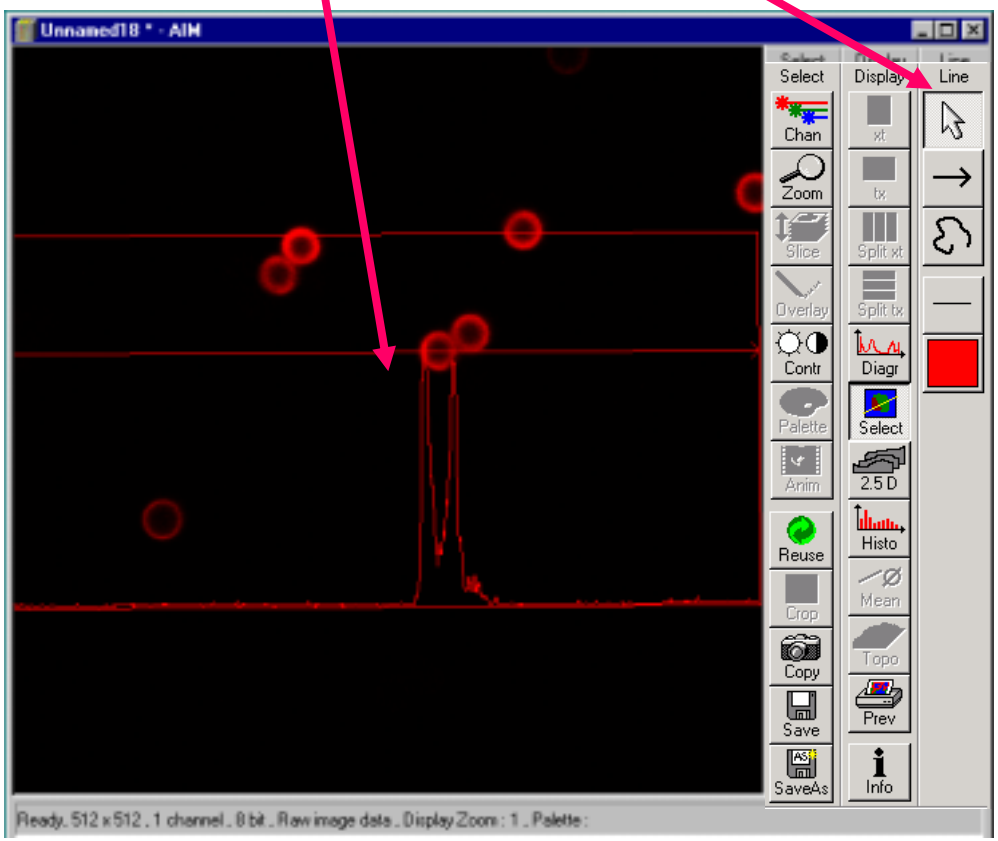
For more or less sections - adjust *Num Slices*

Z - Series Using “Z Sectioning”

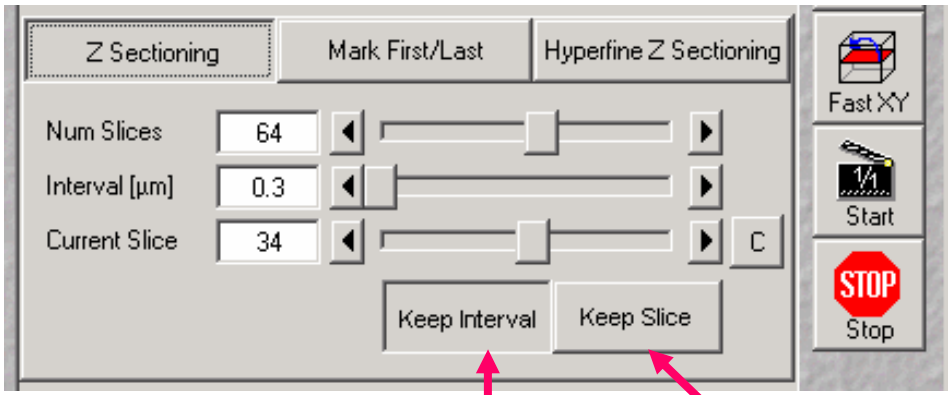


1. Select *Z Stack*
2. Select *Z Sectioning*
3. Select *Line Sel*
4. Select the large arrow button and position the XZ cut line

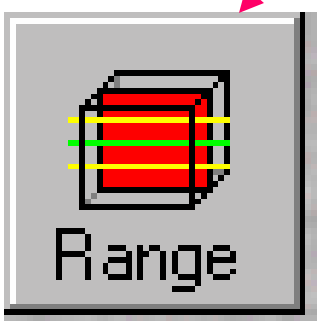


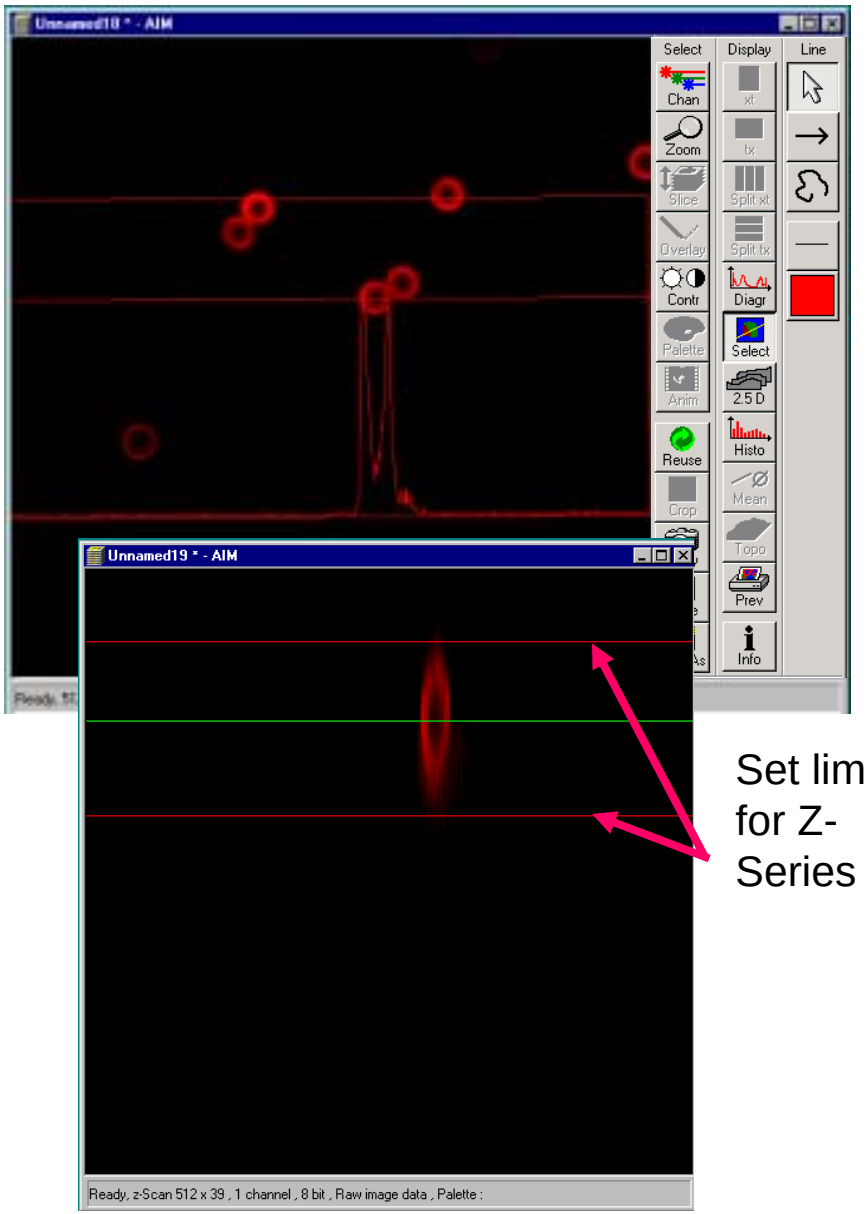


“Z Sectioning” - Setting Range



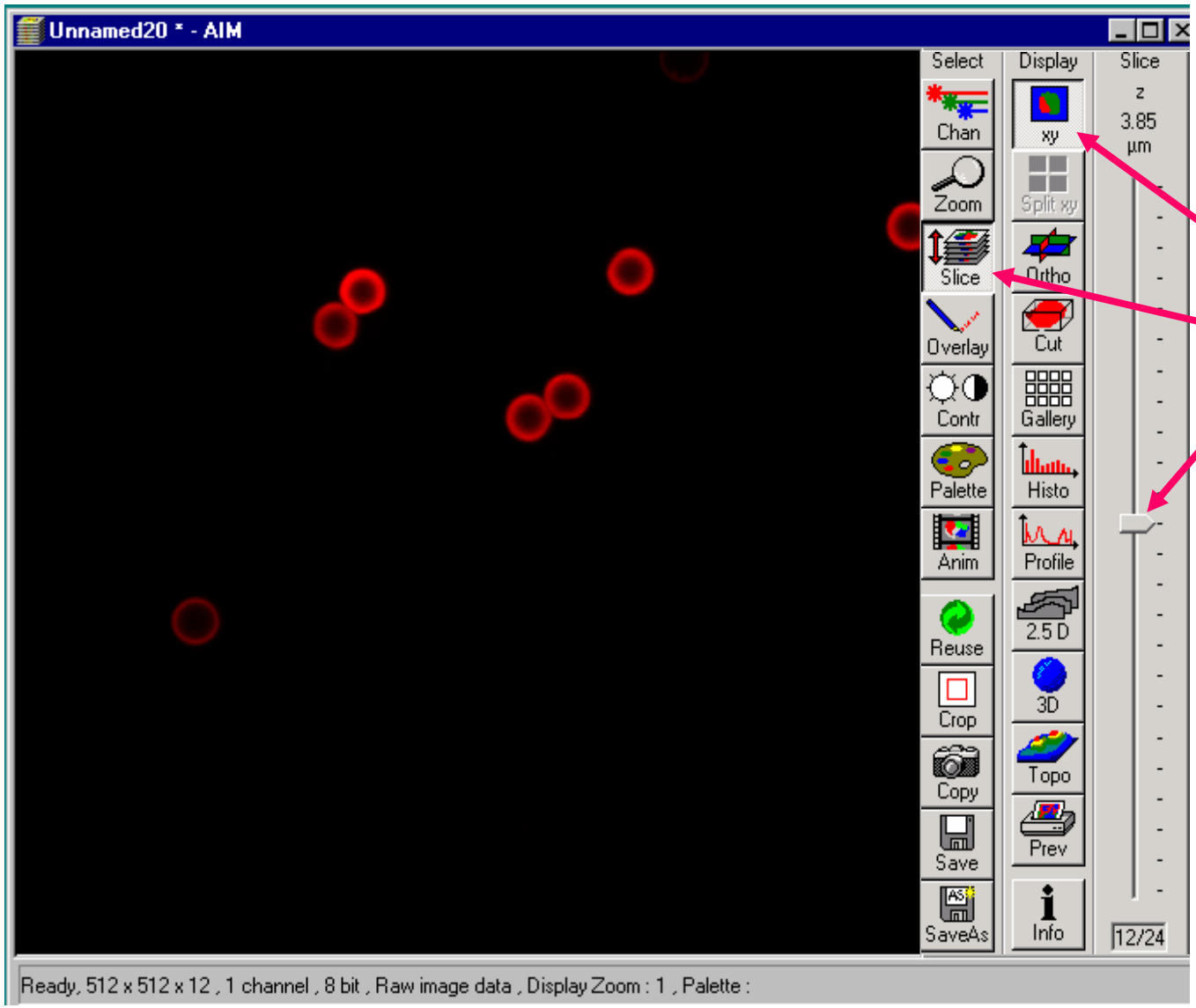
1. Decide whether to *Keep Interval* or *Keep Slices*
2. Select “Range” and position bars to decide where the Z - series begins and ends
3. Select “Start”





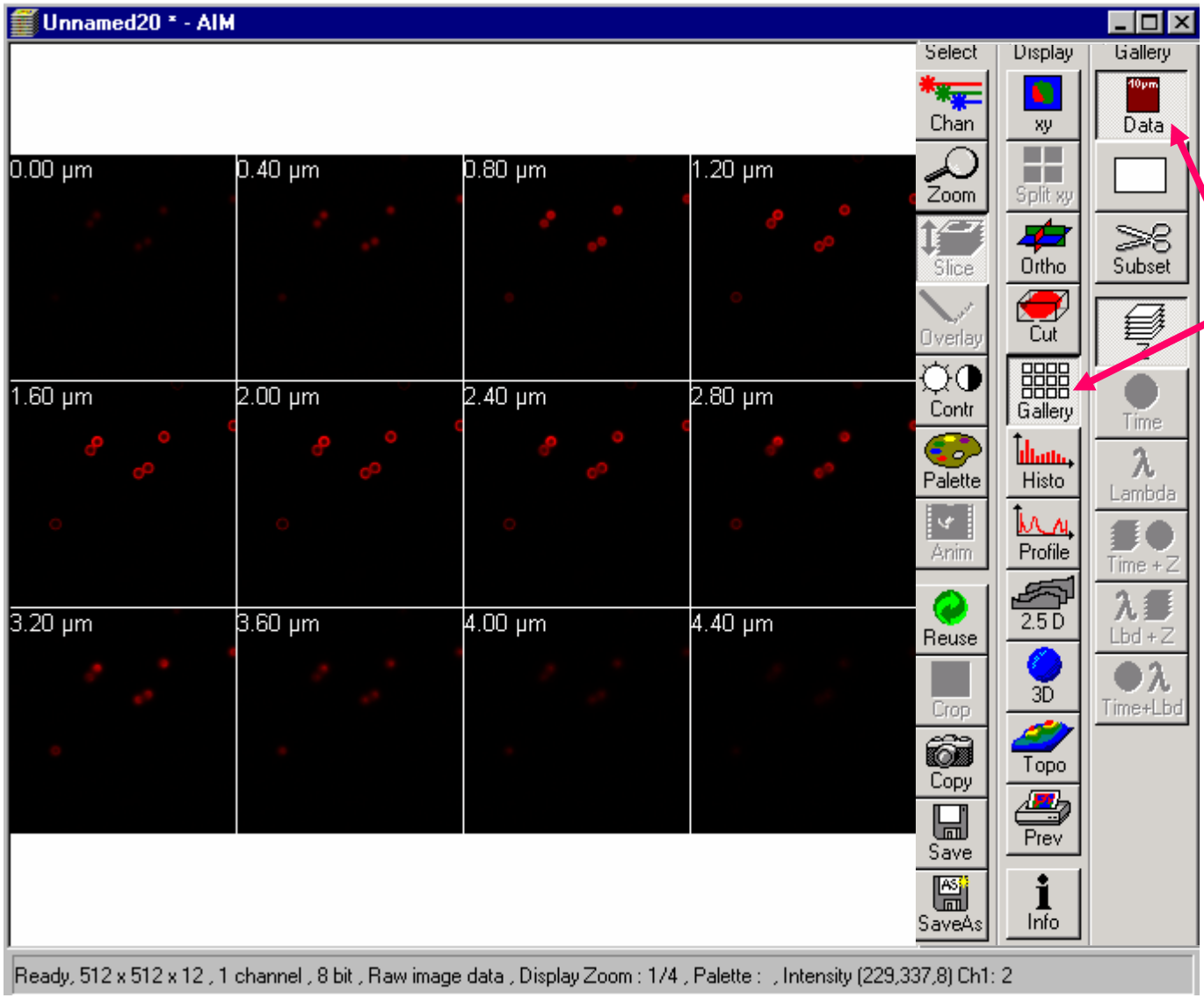
Set limits for Z-Series

Viewing A Z - Series



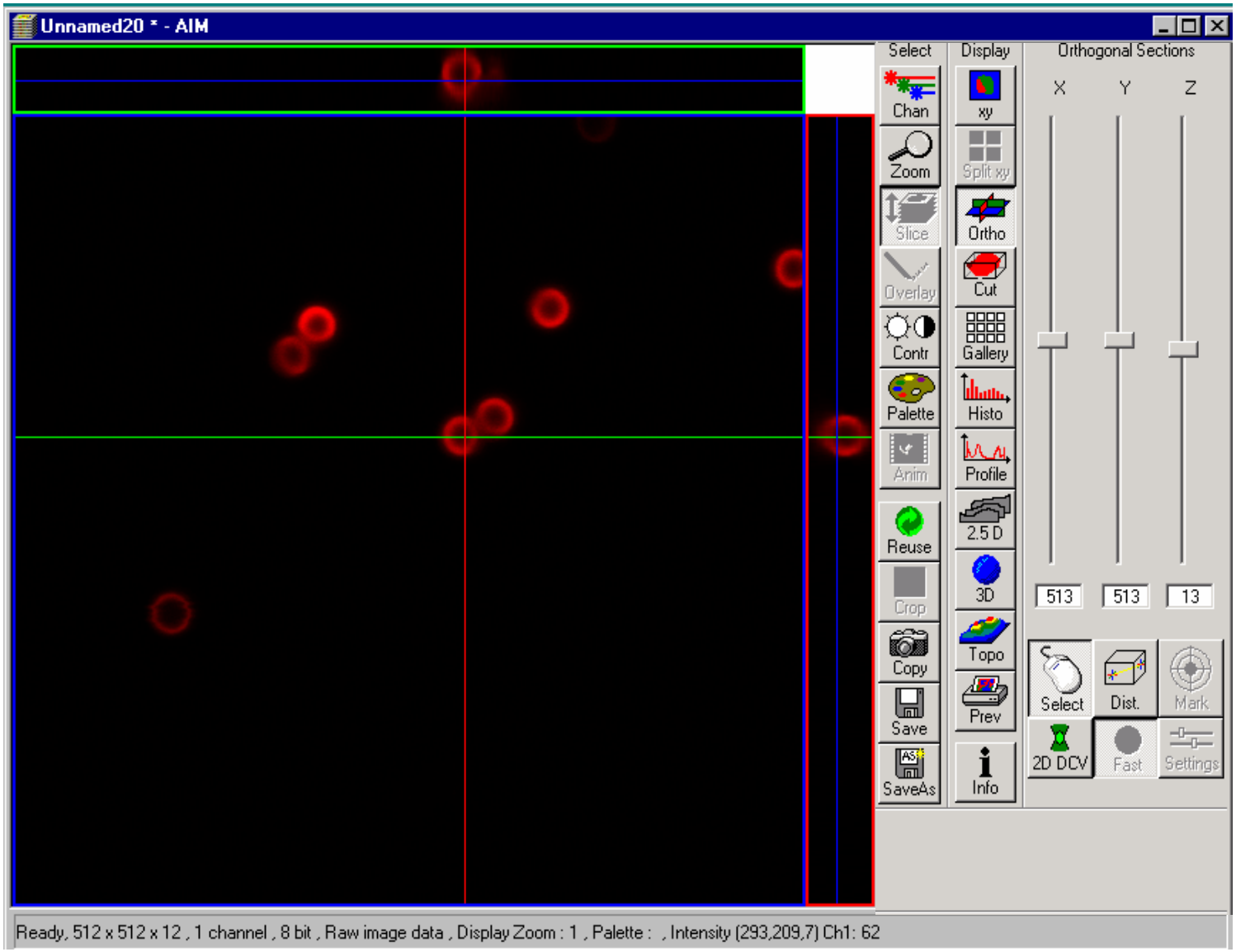
1. Select “xy”
2. Select “Slice”
3. Use scroll bar to view individual sections

Viewing A Z - Series Using Gallery



1. Select *Gallery*
2. Select *Data* for scale
   Use *Subset* to extract sections

Viewing Z- Series Using Orthogonal Sections

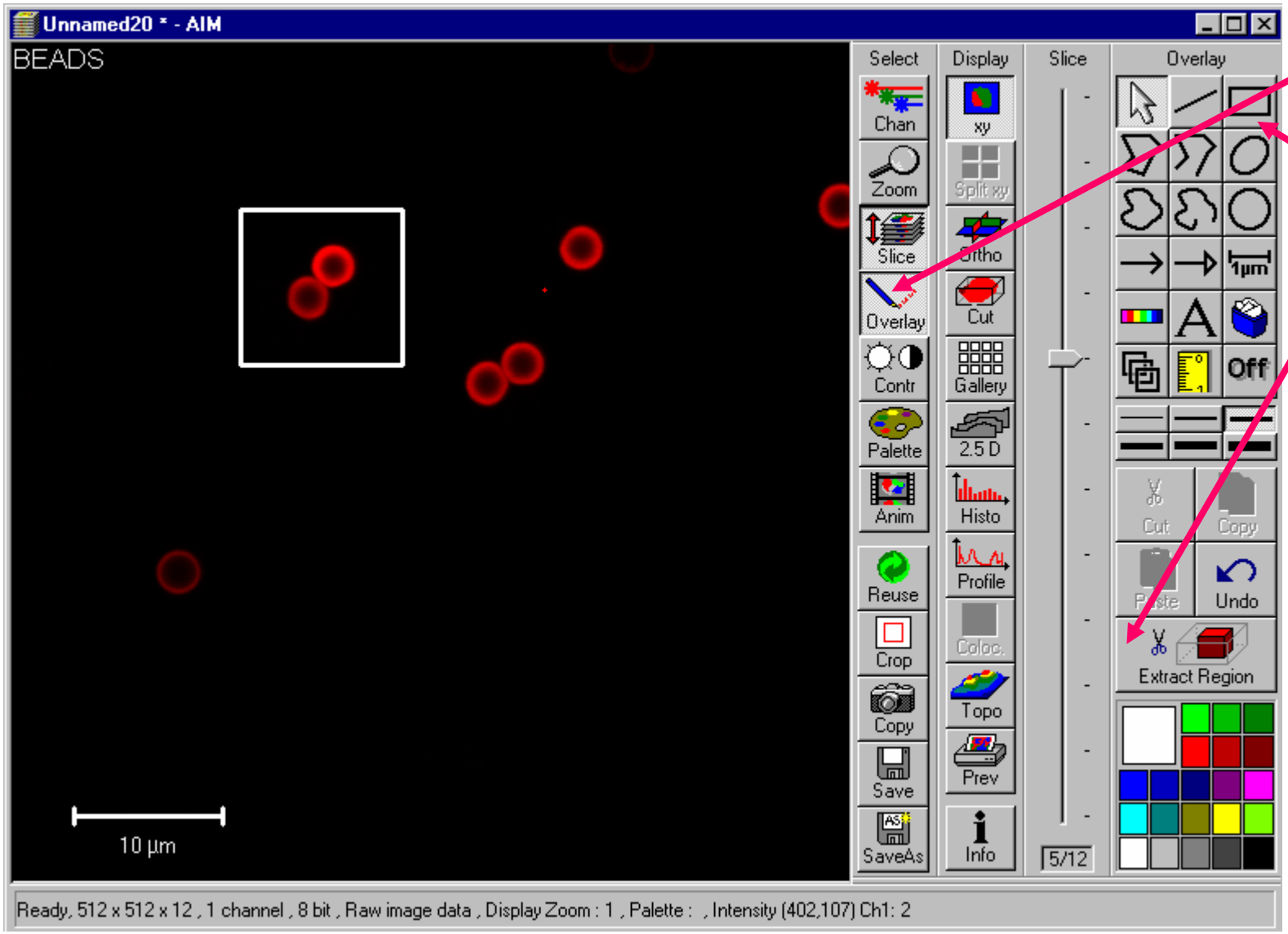


1. Select *Ortho*
2. Select mouse (*Select*)

Using the mouse, position the cut lines.

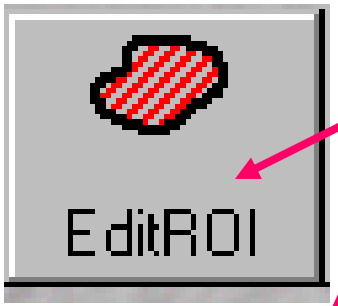
To save orthogonal sections, select *Export* and save as *contents of image window*.

Selecting And Saving A Region Of Interest



1. Select Overlay and define shape for ROI
2. Select “Extract region”
3. Save data

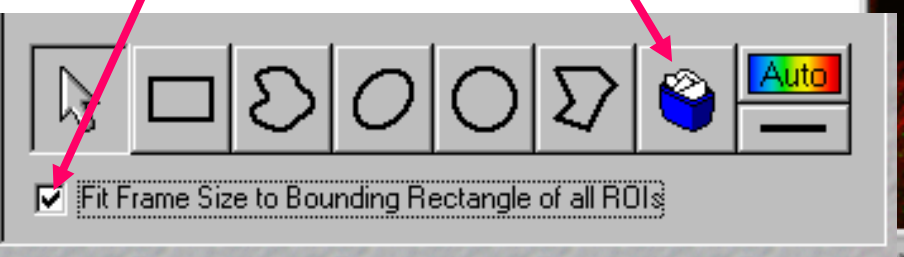
Using “Edit Roi” For Faster Image Acquisition And Data Saving

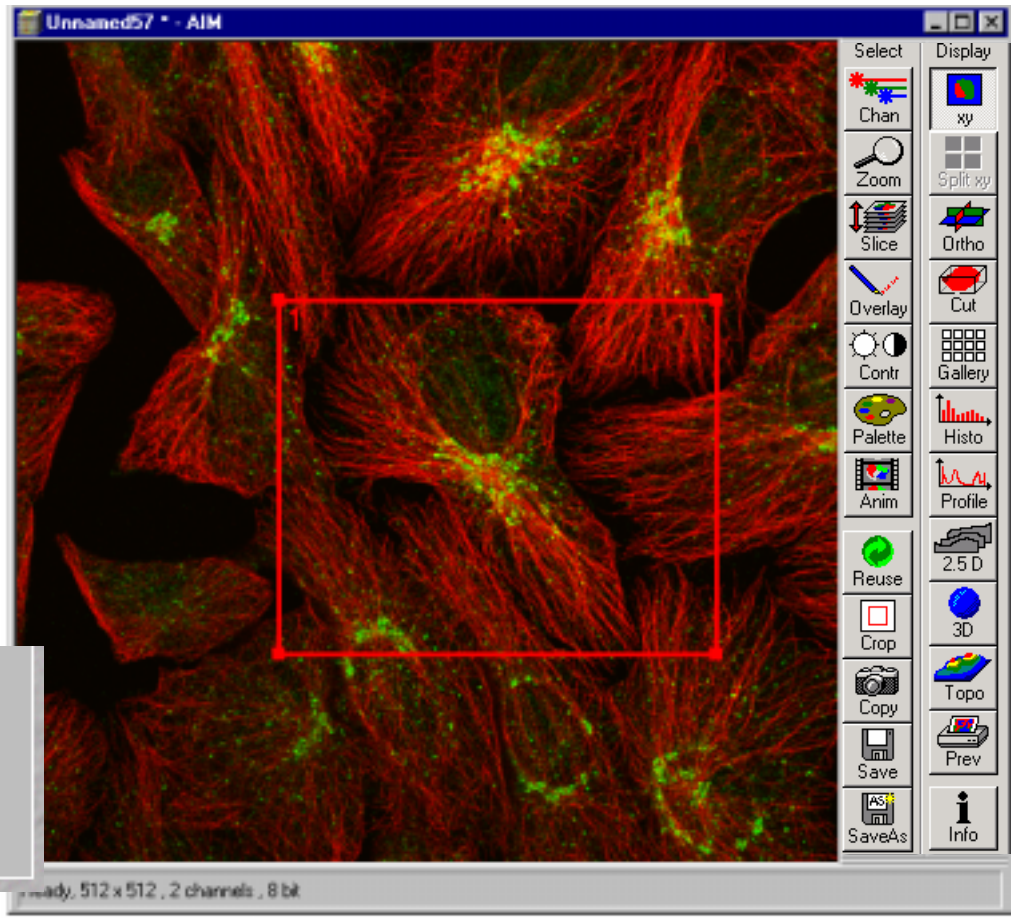


1. Select “EditROI” from the LSM menu bar
2. Select “Fit Frame Size to bounding Rectangle”

1. Choose ROI shape
2. position and size with mouse
3. Scan

To remove ROI select blue bin

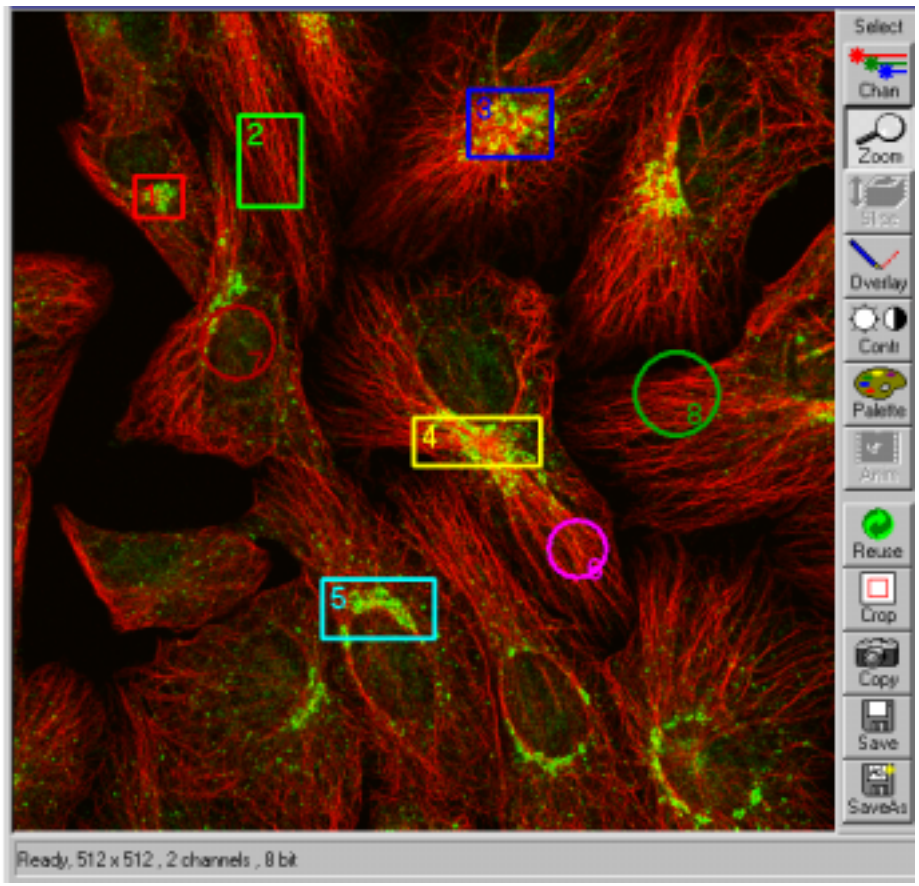


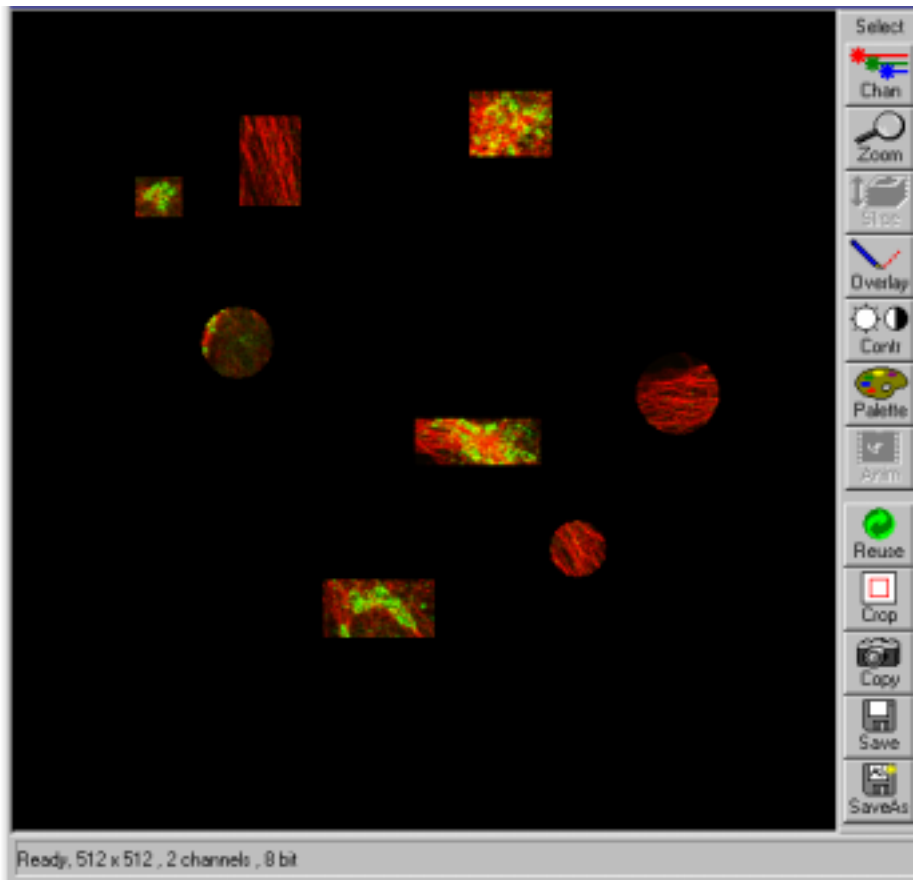


Multiple Regions Of Interest

1. Un-select “Fit Frame Size to bounding Rectangle” Choose ROI shapes
4. Position and size with mouse
5. Scan

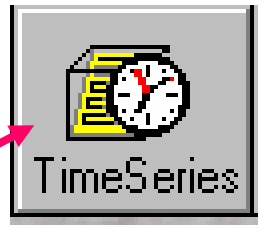
To remove ROI select blue bin

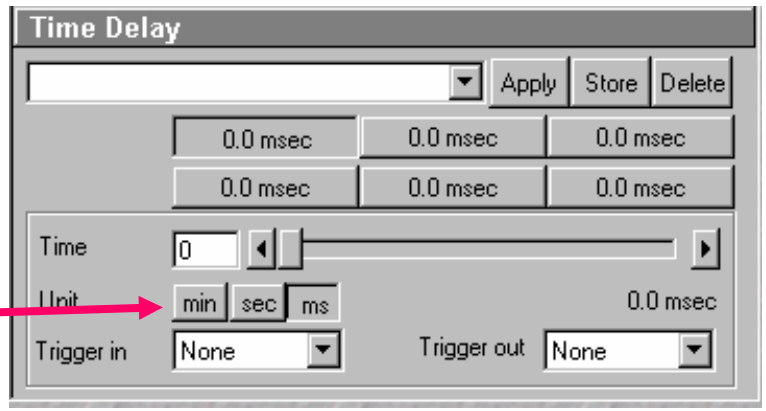


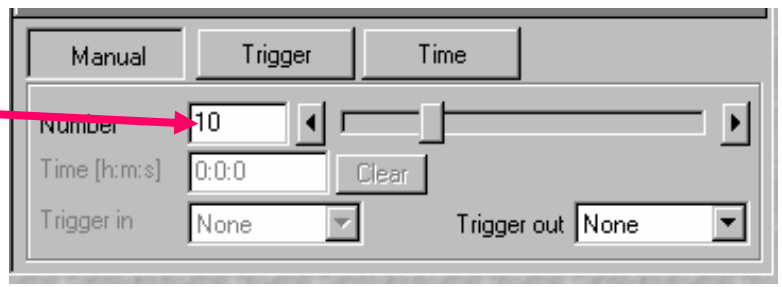


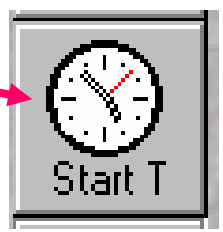
Time Series

1. Set up scanning parameters (Z-Series)
2. Select “Time series” from the LSM menu
3. Select “min,” “sec” or “ms”
4. Enter the number of cycles
5. Select “Start T”



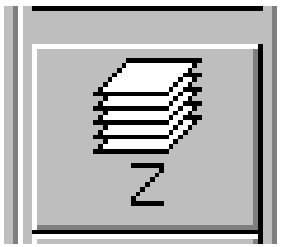


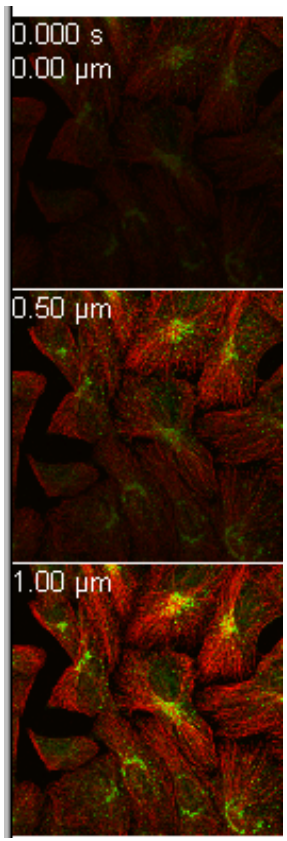




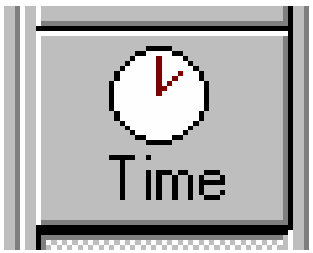
Viewing A Time Series

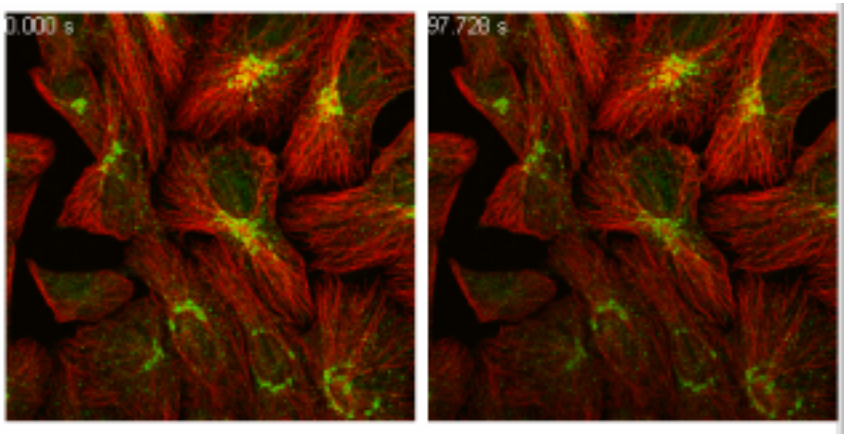
Z Sections for any time



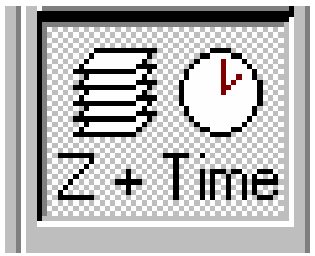


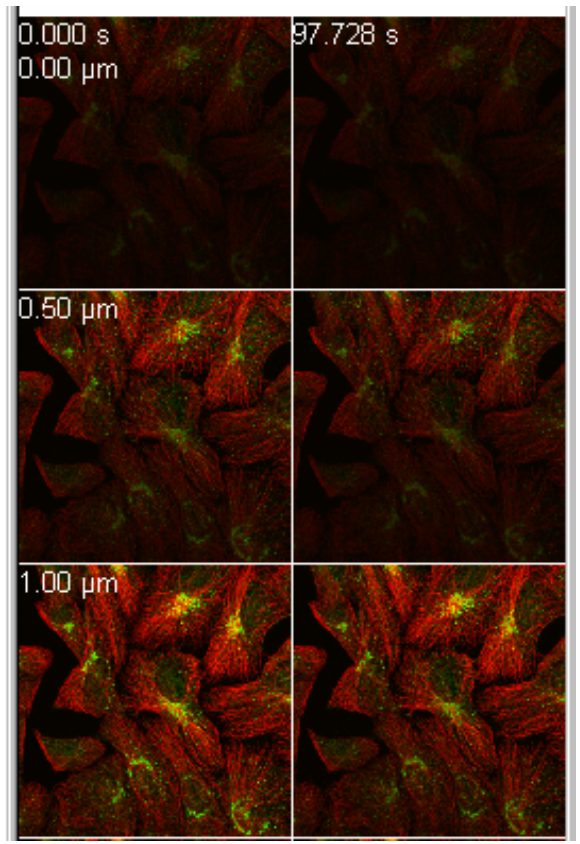
Time points for any Z Section





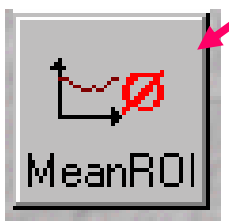
Both Z sections and time series



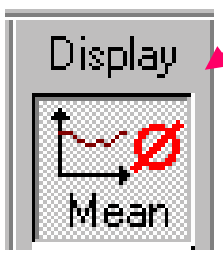


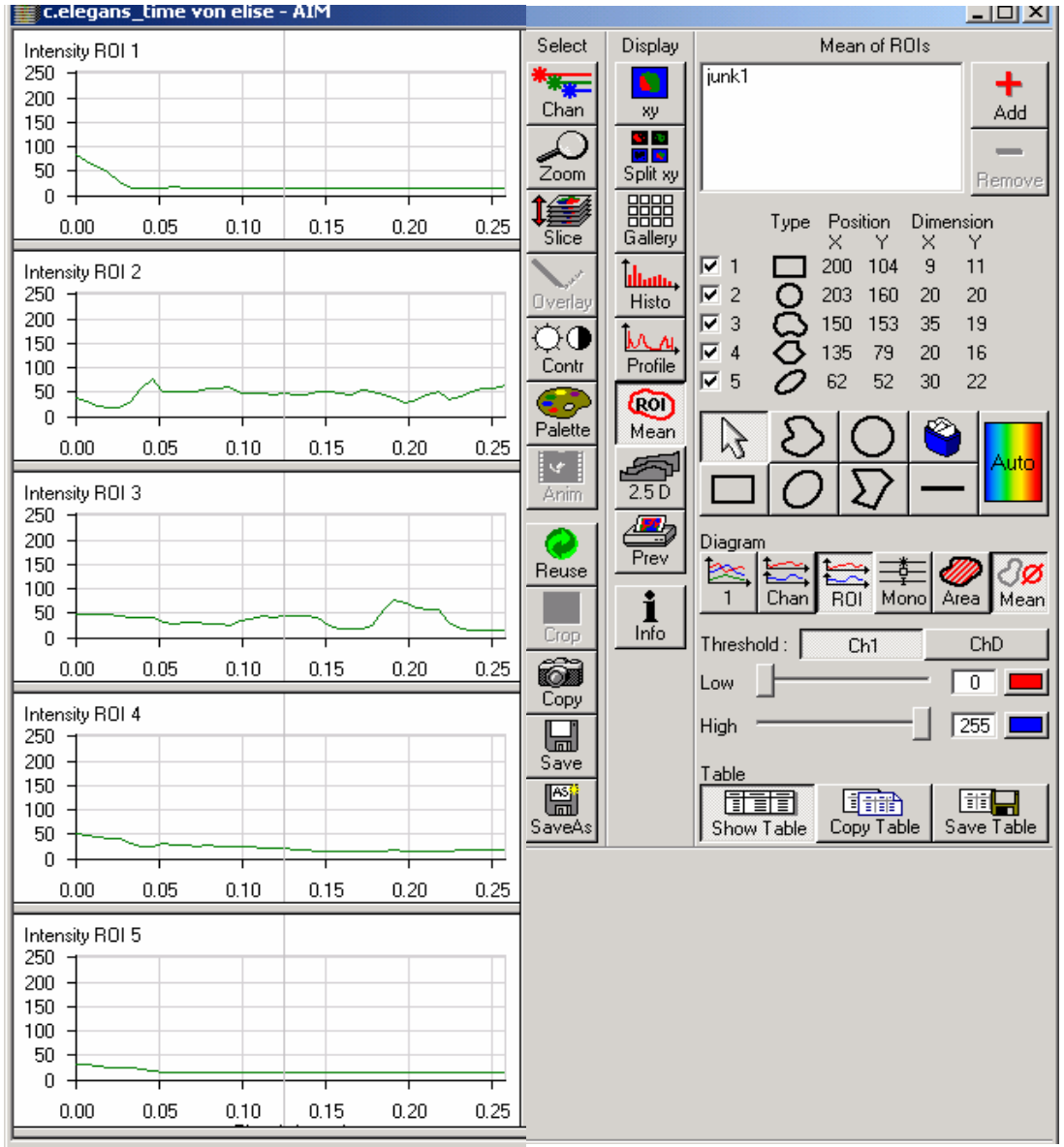
Time Series - Physiology Experiments

1. If required, use multiple regions of interest
2. Set up time series as before
3. Instead of using “TimeSeries”, select “MeanROI” to start scanning

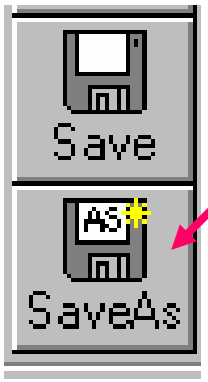


View and save data by selecting

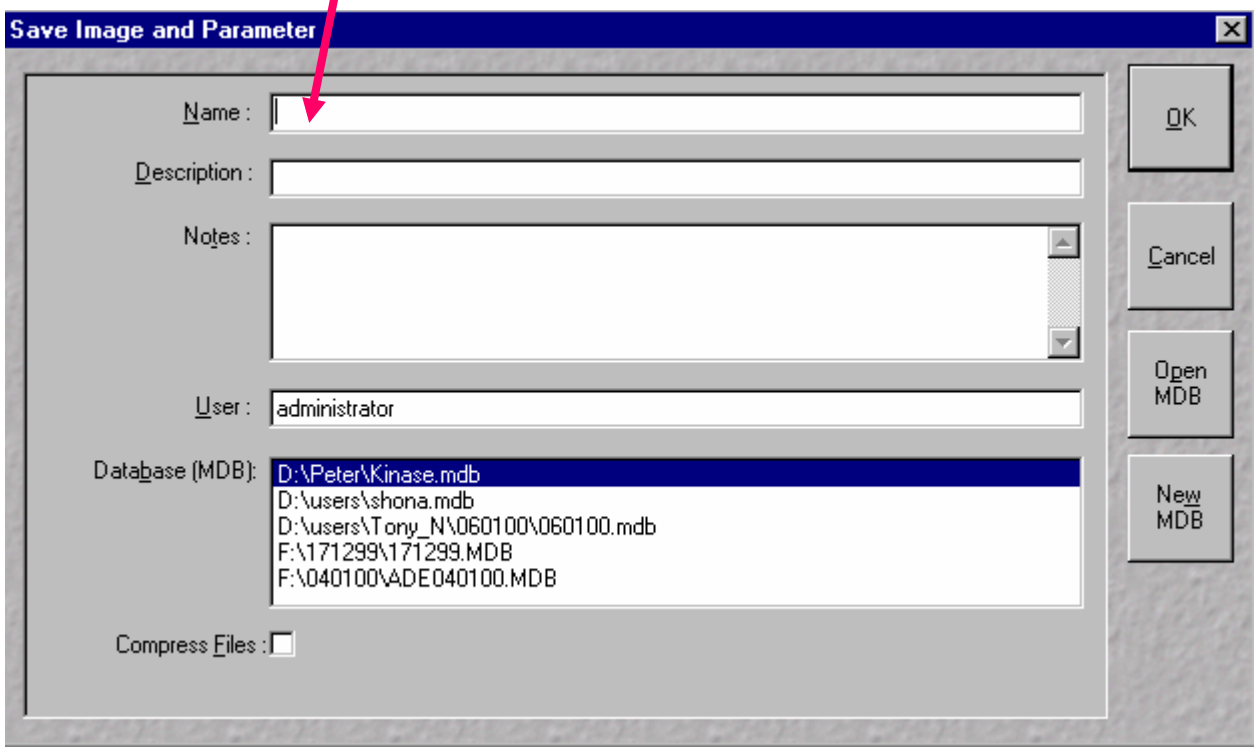




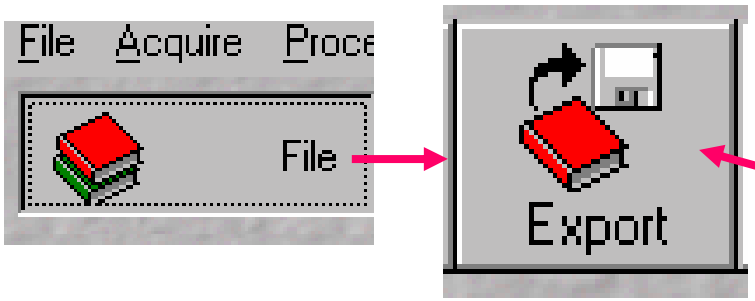
Saving Data - Using Database

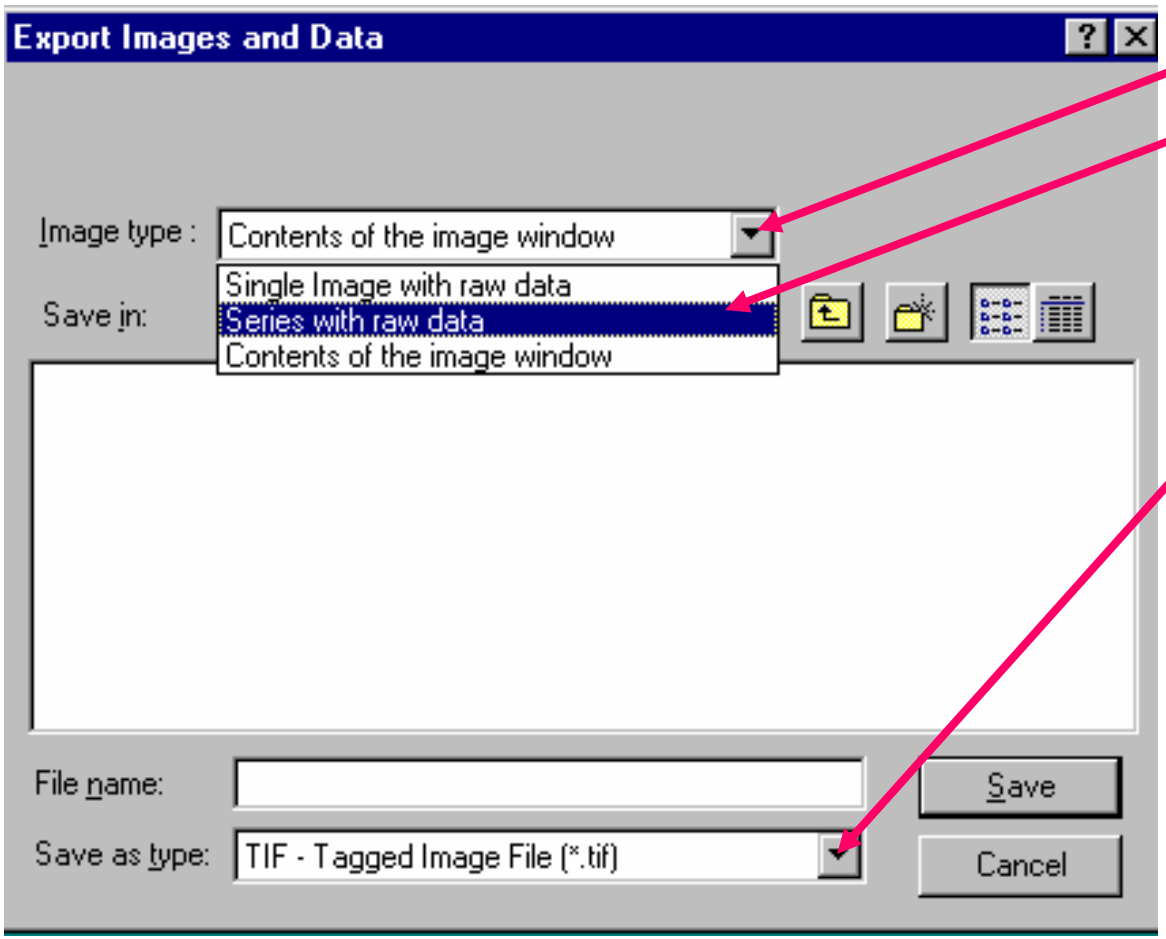


1. Select “Save or “Save as” on image window or LSM menu bar
2. Enter file name and notes if required
3. Select “OK”



SAVING DATA - USING “EXPORT”



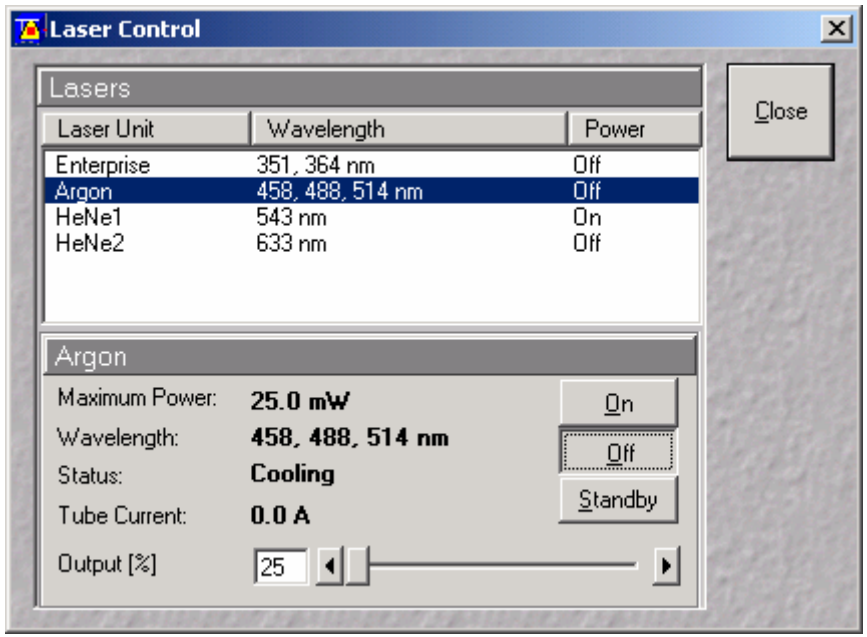


1. Select “File”from LSM menu
2. Select “Export”
3. Select “Image type”
4. Select “Single image with raw data,” “Series with raw data,” or “Contents of image window”
5. Select “Save as type”

“Tif - Tagged image File” is OK for 8 bit
- use “Tiff -16 bit” for 12 bit acquired images (Most other software will not recognize 12 bit)

Shut Down Procedure

1. Acquire - Laser - Switch off lasers



2. File - Exit LSM 510 program
3. START shut down computer operating system.
   You will be warned to wait until the Argon Ion laser has cooled down.
4. Switch off the mercury vapour lamp.
5. Turn off the remote control box but only when the fan on the Argon Ion laser has stopped